



# MANUAL OF BACTERIOLOGY

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## PREFACE TO THE FOURTH EDITION.

IN the present edition the whole subject has been carefully revised. During the five years since the last edition was published, valuable additions to our knowledge have been made in practically every department, whilst in the case of several diseases there have been discoveries of the highest importance. Our object has been to incorporate this new matter and at the same time to maintain the primary object of the work as a text-book for students of medicine. Thus whilst we have dealt with all the facts having a direct bearing on clinical medicine we have also given considerable prominence to matters at present under discussion from the scientific point of view. In this way we have endeavoured to give a faithful representation of the subject as it at present stands both in its practical and theoretical aspects. In the case of several diseases which up till recent times have been investigated by purely bacteriological methods there is now considerable evidence that the causal agent is of protozoal nature. Amongst such conditions the most important are those in which spirochætes are concerned, syphilis and the relapsing fevers being outstanding examples. As, however, the exact biological relationships of these organisms are still matters of dispute we have kept



the diseases in question in the original arrangement. In the appendix will be found an additional chapter dealing with trypanosomiasis and allied affections. A number of new illustrations have been added throughout the book, and the bibliography has been brought up to date.

*October 1907.*

## PREFACE TO THE FIRST EDITION.

THE science of Bacteriology has, within recent years, become so extensive, that in treating the subject in a book of this size we are necessarily restricted to some special departments, unless the description is to be of a superficial character. Accordingly, as this work is intended primarily for students and practitioners of medicine, only those bacteria which are associated with disease in the human subject have been considered. We have made it a chief endeavour to render the work of practical utility for beginners, and, in the account of the more important methods, have given elementary details which our experience in the practical teaching of the subject has shown to be necessary.

In the systematic description of the various bacteria, an attempt has been made to bring into prominence the evidence of their having an etiological relationship to the corresponding diseases, to point out the general laws governing their action as producers of disease, and to consider the effects in particular instances of various modifying circumstances. Much research on certain subjects is so recent that conclusions on many points must necessarily be of a tentative character. We have, therefore, in our statement of results aimed at drawing a distinction between what is proved and what is only probable.

In an Appendix we have treated of four diseases; in two of these the causal organism is not a bacterium, whilst in the other two its nature is not yet determined. These diseases have been

# CONTENTS

|  | PAGE |
|--|------|
| of sections—Staining principles—Mordants and decolorisers—Formulae of stains—Gram's method and its modifications—Stain for tubercle and other acid-fast bacilli—Staining of spores and flagella—The Romanowsky stains—Observation of agglutination and sedimentation—Method of measuring the phagocytic capacity of the leucocytes—Routine bacteriological examination—Methods of inoculation—Autopsies on animals | 85   |

## CHAPTER IV.

### BACTERIA IN AIR, SOIL, AND WATER. ANTISEPTICS.

|   |     |
|---|-----|
| Air: Methods of examination—Results. Soil: Methods of Examination—Varieties of bacteria in soil. Water: Methods of examination—Bacteria in water—Bacterial treatment of sewage. Antiseptics: Methods of investigation—The action of antiseptics—Certain particular antiseptics. | 126 |
|---|-----|

## CHAPTER V.

### RELATIONS OF BACTERIA TO DISEASE—THE PRODUCTION OF TOXINS BY BACTERIA.

|  |     |
|--|-----|
| Introductory—Conditions modifying pathogenicity—Modes of bacterial action—Tissue changes produced by bacteria—Local lesions—General lesions—Disturbance of metabolism by bacterial action—The production of toxins by bacteria, and the nature of these—Allied vegetable and animal poisons—The theory of toxic action | 149 |
|--|-----|

## CHAPTER VI.

### INFLAMMATORY AND SUPPURATIVE CONDITIONS.

|  |     |
|--|-----|
| The relations of inflammation and suppuration—The bacteria of inflammation and suppuration—Experimental inoculation—Lesions in the human subject—Mode of entrance and spread of pyogenic bacteria—Ulcerative endocarditis—Acute suppurative periostitis—Erysipelas—Conjunctivitis—Acute rheumatism—Vaccination treatment of infections by the pyogenic cocci—Methods of examination in inflammatory and suppurative conditions | 172 |
|--|-----|

# CONTENTS

xi

## CHAPTER VII.

### INFLAMMATORY AND SUPPURATIVE CONDITIONS, *CONTINUED* : THE ACUTE PNEUMONIAS, EPIDEMIC CEREBRO-SPINAL MENINGITIS.

|  | PAGE |
|--|------|
| Introductory — Historical — Bacteria in pneumonia — Fraenkel's pneumococcus — Friedlaender's pneumococcus — Distribution of pneumobacteria — Experimental inoculation — Pathology of pneumococcus — Methods of examination. Epidemic cerebro-spinal meningitis . . . . . | 196  |

## CHAPTER VIII.

### GONORRHOEA, SOFT SORE, SYPHILIS.

|  |     |
|--|-----|
| The gonococcus — Microscopical characters — Cultivation — Relations to the disease — Its toxin — Distribution — Gonococcus in joint affections — Methods of diagnosis — Soft sore — Syphilis — Spirochæte pallida — Transmission of the disease to animals . . . . . | 219 |
|--|-----|

## CHAPTER IX.

### TUBERCULOSIS.

|  |     |
|--|-----|
| Historical — Tuberculosis in animals — Tubercle bacillus — Staining reactions — Cultivation of tubercle bacillus — Powers of resistance — Action on the tissues — Histology of tuberculous nodules — Distribution of bacilli — Bacilli in tuberculous discharges — Experimental inoculation — Varieties of tuberculosis — Other acid-fast bacilli — Action of dead tubercle bacilli — Sources of human tuberculosis — Toxins of the tubercle bacillus — Koch's tuberculin — Active immunisation against the tubercle bacillus — Koch's Tuberculin-R — Agglutinative phenomena — Methods of examination . . . . . | 235 |
|--|-----|

## CHAPTER X.

### LEPROSY.

|  |     |
|--|-----|
| Pathological changes — Bacillus of leprosy — Position of the bacilli — Relations to the disease — Methods of diagnosis . . . . . | 267 |
|--|-----|

## CHAPTER XI.

## GLANDERS AND RHINOSCLEROMA.

|   | PAGE |
|---|------|
| Glanders: The natural disease—The glanders bacillus—Cultivation of glanders bacillus—Powers of resistance—Experimental inoculation—Action on the tissues—Mode of spread—Mallein and its preparation—Methods of examination. Rhinoscleroma | 275  |

## CHAPTER XII.

## ACTINOMYCOSIS AND ALLIED DISEASES.

|  |     |
|--|-----|
| Characters of the actinomycetes—Tissue lesions—Distribution of lesions—Cultivation of actinomycetes—Varieties of actinomycetes and allied forms—Experimental inoculation—Methods of examination and diagnosis—Madura disease . . . . . | 286 |
|--|-----|

## CHAPTER XIII.

## ANTHRAX.

|   |     |
|---|-----|
| Historical summary—Bacillus anthracis—Appearances of cultures—Biology—Sporulation—Natural anthrax in animals—Experimental anthrax—Anthrax in man—Pathology—Toxins of the bacillus anthracis—Mode of spread in nature—Immunisation of animals against anthrax—Methods of examination | 300 |
|---|-----|

## CHAPTER XIV.

TYPHOID FEVER—BACILLI ALLIED TO THE TYPHOID  
BACILLUS.

|  |     |
|--|-----|
| Bacillus typhosus—Morphological characters—Characters of cultures—Bacillus coli communis—Reactions of b. typhosus and b. coli—Pathological changes in typhoid fever—Suppuration in typhoid fever—Pathogenic effects produced in animals—The toxic products of typhoid bacillus—Immunisation of animals—Relations of bacilli to the disease—Paratyphoid bacillus—Bacillus enteritidis (Gaertner)—Psittacosis bacillus—Serum diagnosis—Vaccination against typhoid—Methods of examination—Bacteria in dysentery—Bacillus enteritidis sporogenes—Summer diarrhoea . . . . . | 319 |
|--|-----|

# CONTENTS

xiii

## CHAPTER XV.

### DIPHTHERIA.

|  | PAGE |
|--|------|
| Historical—General facts—Bacillus diphtheriæ—Microscopical characters—Distribution—Cultivation—Inoculation experiments—The toxins of diphtheria—Variations in virulence of bacilli—Bacilli allied to the diphtheria bacillus—Summary of pathogenic action—Methods of diagnosis . . . . . | 352  |

## CHAPTER XVI.

### TETANUS.

|  |     |
|--|-----|
| Introductory—Historical—Bacillus tetani—Isolation of bacillus tetani—Characters of cultures—Conditions of growth—Pathogenic effects—Experimental inoculation—Tetanus toxins—Antitetanic serum—Methods of examination—Malignant œdema—Characters of bacillus—Experimental inoculation—Methods of diagnosis—Bacillus botulinus—Quarter - evil—Bacillus ærogenes capsulatus . . . . . | 371 |
|--|-----|

## CHAPTER XVII.

### CHOLERA.

|  |     |
|--|-----|
| Introductory—The cholera spirillum—Distribution of the spirilla—Cultivation—Powers of resistance—Experimental inoculation—Toxins of cholera spirillum—Inoculation of human subject—Immunity—Methods of diagnosis—General summary—Other spirilla resembling the cholera organism—Metchnikoff's spirillum—Finkler and Prior's spirillum—Deneke's spirillum . . . . . | 399 |
|--|-----|

## CHAPTER XVIII.

### INFLUENZA, PLAGUE, RELAPSING FEVER, MALTA FEVER, YELLOW FEVER.

|   |
|---|
| <i>Influenza bacillus</i> —Microscopical characters—Cultivation—Distribution—Experimental inoculation—Methods of examina- |
|---|

|   |     |
|---|-----|
| tion— <i>Bacillus of plague</i> —Microscopical characters—Cultivation—Anatomical changes produced and distribution of bacilli—Experimental inoculation—Paths and mode of infection—Toxins, immunity, etc.—Methods of diagnosis— <i>Relapsing fever and African tick fever</i> —Characters of the spirillum—Relations to the disease—Immunity—African tick fever— <i>Malta fever</i> — <i>Micrococcus melitensis</i> —Relations to the disease—Mode of spread of the disease—Methods of diagnosis— <i>Yellow fever</i> —Etiology of yellow fever . . . . . | 420 |
|---|-----|

## CHAPTER XIX.

## IMMUNITY.

|   |     |
|---|-----|
| Introductory—Acquired immunity—Artificial immunity—Varieties—Active immunity—Methods of production—Attenuation and exaltation of virulence—Passive immunity—Action of the serum—Antitoxic serum—Standardising of toxins and of antisera—Nature of antitoxic action—Ehrlich's theory of the constitution of toxins—Antibacterial serum—Bactericidal and lysogenic action—Hæmolytic and other sera—Methods of hæmolytic tests—Opsonic action—Agglutination—Precipitins—Therapeutic effects of anti-sera—Theories as to acquired immunity—Ehrlich's side-chain theory—Serum anaphylaxis—Theory of phagocytosis—Natural immunity—Natural bactericidal powers—Natural susceptibility to toxins . . . . . | 456 |
|---|-----|

## APPENDIX A.

## SMALLPOX AND VACCINATION.

|   |     |
|---|-----|
| Jennerian vaccination—Relationship of smallpox to cowpox—Micro-organisms associated with smallpox—The nature of vaccination . . . . . | 503 |
|---|-----|

## APPENDIX B.

## HYDROPHOBIA.

|   |     |
|---|-----|
| Introductory—Pathology—The virus of hydrophobia—Prophylaxis—Antirabic serum—Methods . . . . . | 510 |
|---|-----|

# CONTENTS

xv

## APPENDIX C.

### MALARIAL FEVER.

|  | PAGE |
|--|------|
| The malarial parasite—The cycle of the malarial parasite in man  |      |
| —The cycle in the mosquito—Varieties of the malarial parasite—General considerations—The pathology of malaria— |      |
| Methods of examination . . . . .   | 521  |

## APPENDIX D.

### AMŒBIC DYSENTERY.

|  |     |
|--|-----|
| Amœbic dysentery—Characters of the amœba—Distribution of the amœbæ—Experimental inoculation—Methods of examination | 537 |
|--|-----|

## APPENDIX E.

### TRYPANOSOMIASIS—KÁLA-ÁZAR—PIROPLASMOSIS.

|  |     |
|--|-----|
| <i>The pathogenic trypanosomes</i> —General morphology of the trypanosomata—Trypanosoma Lewisi—Nagana or tse-tse fly disease—Trypanosoma of sleeping sickness—Trypanosoma gambiense—Kála-azar—Dehli sore— <i>Piroplasmosis</i> . . . . . | 544 |
|--|-----|

|                        |     |
|------------------------|-----|
| BIBLIOGRAPHY . . . . . | 571 |
|------------------------|-----|

|                 |     |
|-----------------|-----|
| INDEX . . . . . | 593 |
|-----------------|-----|





# LIST OF ILLUSTRATIONS.

| FIG.   | PAGE |
|--|------|
| 1. Forms of bacteria . . . . .   | 13   |
| 2. Hot-air steriliser . . . . .  | 27   |
| 3. Koch's steam steriliser . . . . .                                       | 27   |
| 4. Autoclave . . . . .   | 29   |
| 5. Steriliser for blood serum . . . . .                                    | 30   |
| 6. Meat press . . . . .  | 31   |
| 7. Hot-water funnel . . . . .  | 35   |
| 8. Blood serum inspissator . . . . .                                       | 40   |
| 9. Potato jar . . . . .  | 45   |
| 10. Cylinder of potato cut obliquely . . . . .                             | 45   |
| 11. Ehrlich's tube containing piece of potato . . . . .                    | 45   |
| 12. Apparatus for filling tubes . . . . .                                  | 48   |
| 13. Tubes of media . . . . .   | 48   |
| 14. Platinum wires in glass handles . . . . .                              | 49   |
| 15. Method of inoculating solid tubes . . . . .                            | 50   |
| 16. Rack for platinum needles . . . . .                                    | 50   |
| 17. Petri's capsule . . . . .  | 51   |
| 18. Koch's levelling apparatus for use in preparing plates . . . . .       | 54   |
| 19. Koch's levelling apparatus . . . . .                                   | 54   |
| 20. Esmarch's tube for roll culture . . . . .                              | 55   |
| 21. Apparatus for supplying hydrogen for anaerobic cultures . . . . .      | 58   |
| 22. Esmarch's roll-tube adapted for culture containing anaerobes . . . . . | 59   |
| 23. Bulloch's apparatus for anaerobic plate cultures . . . . .             | 59   |
| 24. Flask for anaerobes in liquid media . . . . .                          | 61   |
| 25. Flask arranged for culture of anaerobes which develop gas . . . . .    | 62   |
| 26. Tubes for anaerobic cultures on the surface of solid media . . . . .   | 62   |
| 27. Slides for hanging-drop cultures . . . . .                             | 63   |
| 28. Graham Brown's chamber for anaerobic hanging-drops . . . . .           | 64   |
| 29. Apparatus for counting colonies . . . . .                              | 65   |
| 30. Wright's 250 c.mm. pipette fitted with nipple . . . . .                | 66   |
| 31. Geissler's vacuum pump for filtering cultures . . . . .                | 70   |
| 32. Chamberland's candle and flask arranged for filtration . . . . .       | 70   |

| FIG.  | PAGE |
|---|------|
| 33. Chamberland's bougie with lamp funnel . . . . .   | 71   |
| 34. Bougie inserted through rubber stopper . . . . .  | 71   |
| 35. Muencke's modification of Chamberland's filter . . . . .  | 72   |
| 36. Flask fitted with porcelain bougie for filtering large quantities<br>of fluid . . . . .                       | 73   |
| 37. Flask for filtering small quantities of fluid . . . . .   | 73   |
| 38. Tubes for demonstrating gas-formation by bacteria . . . . .   | 76   |
| 39. Geryk air-pump for drying <i>in vacuo</i> . . . . .   | 79   |
| 40. Reichert's gas regulator . . . . .  | 80   |
| 41. Hearson's incubator for use at 37° C. . . . .   | 81   |
| 42. Cornet's forceps for holding cover-glasses . . . . .  | 87   |
| 43. Needle with square of paper on end for manipulating paraffin<br>sections . . . . .                            | 92   |
| 44. Syphon wash-bottle for distilled water . . . . .  | 96   |
| 45. Wright's 5 c.mm. pipette . . . . .  | 108  |
| 46. Tubes used in testing agglutinating and sedimenting properties<br>of serum . . . . .                          | 110  |
| 47. Wright's blood-capsule . . . . .  | 114  |
| 48. Test-tube and pipette arranged for obtaining fluids containing<br>bacteria . . . . .                          | 116  |
| 49. Hollow needle for intraperitoneal inoculations . . . . .  | 121  |
| 50. Hesse's tube . . . . .  | 127  |
| 51. Petri's sand filter . . . . .   | 128  |
| 52. Staphylococcus pyogenes aureus, young culture on agar.<br>× 1000 . . . . .                                    | 175  |
| 53. Two stab cultures of staphylococcus pyogenes aureus in gelatin . . . . .                                      | 175  |
| 54. Streptococcus pyogenes, young culture on agar. × 1000 . . . . .   | 176  |
| 55. Culture of the streptococcus pyogenes on an agar plate . . . . .  | 177  |
| 56. Bacillus pyocyaneus ; young culture on agar. × 1000 . . . . .   | 177  |
| 57. Micrococcus tetragenus. × 1000 . . . . .  | 181  |
| 58. Streptococci in acute suppuration. × 1000 . . . . .   | 184  |
| 59. Minute focus of commencing suppuration in brain. × 50 . . . . .   | 186  |
| 60. Secondary infection of a glomerulus of kidney by the staphylo-<br>coccus aureus. × 300 . . . . .              | 187  |
| 61. Section of a vegetation in ulcerative endocarditis. × 600 . . . . .   | 189  |
| 62. Film preparation from a case of acute conjunctivitis, showing<br>the Koch-Weeks bacilli. × 1000 . . . . .     | 192  |
| 63. Film preparation of conjunctival secretion showing the diplo-<br>bacillus of conjunctivitis. × 1000 . . . . . | 192  |
| 64. Film preparation of pneumonic sputum, showing numerous<br>pneumococci (Fraenkel's). × 1000 . . . . .          | 199  |
| 65. Friedländer's pneumobacillus, from exudate in a case of<br>pneumonia. × 1000 . . . . .                        | 200  |
| 66. Fraenkel's pneumococcus in serous exudation. × 1000 . . . . .   | 200  |
| 67. Stroke culture of Fraenkel's pneumococcus on blood agar . . . . .   | 201  |

# LIST OF ILLUSTRATIONS

xix

| FIG.   | PAGE |
|--|------|
| 68. Fraenkel's pneumococcus from a pure culture on blood agar.<br>× 1000 . . . . .   | 202  |
| 69. Stab culture of Friedländer's pneumobacillus . . . . .   | 203  |
| 70. Friedländer's pneumobacillus, from a young culture on agar.<br>× 1000 . . . . .  | 203  |
| 71. Capsulated pneumococci in blood taken from the heart of a<br>rabbit. × 1000 . . . . .  | 206  |
| 72. Film preparation of exudation from a case of meningitis. × 1000  | 213  |
| 73. Pure culture of diplococcus intracellularis . . . . .  | 214  |
| 74. Portion of film of gonorrhœal pus. × 1000 . . . . .  | 220  |
| 75. Gonococci, from a pure culture on blood agar. × 1000 . . . . .   | 221  |
| 76. Film preparations of pus from soft chancre, showing Ducrey's<br>bacillus. × 1500 . . . . .   | 227  |
| 77. Ducrey's bacillus. × 1500 . . . . .  | 228  |
| 78 and 79. Film preparations from juice of hard chancre showing<br>spirochæte pallida. × 1000 . . . . .  | 230  |
| 80. Section of spleen from a case of congenital syphilis, showing<br>spirochæte pallida. × 1000 . . . . .  | 231  |
| 81. Spirochæte refringens. × 1000 . . . . .  | 231  |
| 82. Tubercle bacilli, from a pure culture on glycerin agar. × 1000   | 237  |
| 83. Tubercle bacilli in phthisical sputum. × 1000 . . . . .  | 238  |
| 84. Cultures of tubercle bacilli on glycerin agar . . . . .  | 240  |
| 85. Tubercle bacilli in section of human lung in acute phthisis.<br>× 1000 . . . . .   | 244  |
| 86. Tubercle bacilli in giant-cells. × 1000 . . . . .  | 245  |
| 87. Tubercle bacilli in urine. × 1000 . . . . .  | 246  |
| 88. Moeller's Timothy-grass bacillus. × 1000 . . . . .   | 253  |
| 89. Cultures of acid-fast bacilli grown at room temperature . . . . .  | 253  |
| 90. Smegma bacilli. × 1000 . . . . .   | 254  |
| 91. Section through leprous skin, showing the masses of cellular<br>granulation tissue in the cutis. × 80 . . . . .  | 268  |
| 92. Superficial part of leprous skin. × 500 . . . . .  | 270  |
| 93. High-power view of portion of leprous nodule showing the<br>arrangement of the bacilli within the cells of the granula-<br>tion tissue. × 1100 . . . . . | 271  |
| 94. Glanders bacilli amongst broken-down cells. × 1000 . . . . .   | 277  |
| 95. Glanders bacilli. × 1000 . . . . .   | 278  |
| 96. Actinomyces of human liver. × 500 . . . . .  | 288  |
| 97. Actinomyces in human kidney. × 500 . . . . .   | 289  |
| 98. Colonies of actinomyces. × 60 . . . . .  | 290  |
| 99. Cultures of the actinomyces on glycerin agar . . . . .   | 293  |
| 100. Actinomyces, from a culture on glycerin agar. × 1000 . . . . .  | 294  |
| 101. Shake cultures of actinomyces in glucose agar . . . . .   | 295  |
| 102. Section of a colony of actinomyces from a culture in blood<br>serum. × 1500 . . . . .   | 295  |

| FIG. |  | PAGE |
|------|--|------|
| 103. | <i>Streptothrix Maduraë</i> . × 1000 . . . . .   | 298  |
| 104. | Surface colony of the anthrax bacillus on an agar plate.<br>× 30 . . . . .   | 302  |
| 105. | Anthrax bacilli, arranged in chains, from a twenty-four<br>hours' culture on agar at 37° C. × 1000 . . . . .                               | 303  |
| 106. | Stab culture of the anthrax bacillus in peptone-gelatin . . . . .  | 303  |
| 107. | Anthrax bacilli containing spores. × 1000 . . . . .  | 305  |
| 108. | Scraping from spleen of guinea-pig dead of anthrax. × 1000 . . . . .   | 307  |
| 109. | Portion of kidney of a guinea-pig dead of anthrax. × 300 . . . . .   | 309  |
| 110. | A large clump of typhoid bacilli in a spleen. × 500 . . . . .  | 320  |
| 111. | Typhoid bacilli, from a young culture on agar, showing some<br>filamentous forms. × 1000 . . . . .   | 321  |
| 112. | Typhoid bacilli, from a young culture on agar, showing<br>flagella. × 1000 . . . . .   | 322  |
| 113. | Culture of the typhoid bacillus and of the bacillus coli . . . . .   | 323  |
| 114. | Colonies of the typhoid bacillus in a gelatin plate. × 15 . . . . .  | 324  |
| 115. | <i>Bacillus coli communis</i> . × 1000 . . . . .   | 325  |
| 116. | Film preparation from diphtheria membrane; showing<br>numerous diphtheria bacilli. × 1000 . . . . .  | 354  |
| 117. | Section through a diphtheritic membrane in trachea, show-<br>ing diphtheria bacilli. × 1000 . . . . .                                      | 355  |
| 118. | Cultures of the diphtheria bacillus on an agar plate . . . . .   | 357  |
| 119. | Diphtheria bacilli from a twenty-four hours' culture on<br>agar. × 1000 . . . . .  | 357  |
| 120. | Diphtheria bacilli, from a three days' agar culture. × 1000 . . . . .  | 358  |
| 121. | Involution forms of the diphtheria bacillus. × 1000 . . . . .  | 358  |
| 122. | Pseudo-diphtheria bacillus (Hofmann's). × 1000 . . . . .   | 366  |
| 123. | Xerosis bacillus from a young agar culture. × 1000 . . . . .   | 367  |
| 124. | Film preparation of discharge from wound in a case of<br>tetanus, showing several tetanus bacilli of "drumstick"<br>form. × 1000 . . . . . | 373  |
| 125. | Tetanus bacilli, showing flagella. × 1000 . . . . .  | 374  |
| 126. | Spiral composed of numerous twisted flagella of the tetanus<br>bacillus. × 1000 . . . . .  | 375  |
| 127. | Tetanus bacilli, some of which possess spores. × 1000 . . . . .  | 375  |
| 128. | Stab culture of the tetanus bacillus in glucose gelatin . . . . .  | 376  |
| 129. | Film preparation from the affected tissues in a case of<br>malignant œdema. × 1000 . . . . .   | 389  |
| 130. | Bacillus of malignant œdema, showing spores. × 1000 . . . . .  | 390  |
| 131. | Stab cultures in agar—tetanus bacillus, bacillus of malignant<br>œdema, and bacillus of quarter-evil . . . . .                             | 391  |
| 132. | Bacillus of quarter-evil, showing spores. × 1000 . . . . .   | 397  |
| 133. | <i>Bacillus aerogenes capsulatus</i> . . . . .   | 398  |
| 134. | Cholera spirilla, from a culture on agar of twenty-four hours'<br>growth. × 1000 . . . . .   | 400  |

# LIST OF ILLUSTRATIONS

xxi

| FIG.  | PAGE |
|---|------|
| 185. Cholera spirilla stained to show the terminal flagella.<br>× 1000 . . . . .  | 401  |
| 136. Cholera spirilla from an old agar culture. × 1000 . . . . .  | 401  |
| 137. Puncture culture of the cholera spirillum . . . . .  | 403  |
| 138. Colonies of the cholera spirillum on a gelatin plate . . . . .   | 404  |
| 139. Metchnikoff's spirillum. × 1000 . . . . .  | 417  |
| 140. Puncture cultures in peptone-gelatin . . . . .   | 418  |
| 141. Finkler and Prior's spirillum. × 1000 . . . . .  | 419  |
| 142. Influenza bacilli from a culture on blood agar. × 1000 . . . . .   | 420  |
| 143. Film preparation from a plague bubo. × 1000 . . . . .  | 426  |
| 144. Bacillus of plague from a young culture on agar. × 1000 . . . . .  | 427  |
| 145. Bacillus of plague in chains. × 1000 . . . . .   | 427  |
| 146. Culture of the bacillus of plague on 4 per cent salt agar.<br>× 1000 . . . . .   | 428  |
| 147. Section of a human lymphatic gland in plague. × 50 . . . . .   | 430  |
| 148. Film preparation of spleen of rat after inoculation with the<br>bacillus of plague. × 1000 . . . . .   | 432  |
| 149. Spirilla of relapsing fever in human blood. × about 1000 . . . . .   | 439  |
| 150. Spirillum Obermeieri in blood of infected mouse. × 1000 . . . . .  | 441  |
| 151. Film of human blood containing spirillum of tick fever.<br>× 1000 . . . . .  | 444  |
| 152. Spirillum of human tick fever ( <i>Spirillum Duttoni</i> ) in blood<br>of infected mouse. × 1000 . . . . .   | 445  |
| 153. <i>Micrococcus melitensis</i> . × 1000 . . . . .   | 448  |
| 154-159. Various phases of the benign tertian parasite . . . . .  | 525  |
| 160-165. Exemplifying phases of the malignant parasite . . . . .  | 526  |
| 166. Amœbæ of dysentery . . . . .   | 538  |
| 167. Section of wall of liver abscess, showing an amœba of spherical<br>form with vacuolated protoplasm. × 1000 . . . . .   | 540  |
| 168. <i>Trypanosoma Brucei</i> from blood of infected rat. Note in two<br>of the organisms commencing division of micronucleus and<br>undulating membrane. × 1000 . . . . . | 554  |
| 169. <i>Trypanosoma gambiense</i> from blood of guinea-pig. × 1000 . . . . .  | 557  |
| 170. Leishman-Donovan bodies from spleen smear. × 1000 . . . . .  | 564  |
| 171. Leishman-Donovan bodies within endothelial cell in spleen.<br>× 1000 . . . . .   | 565  |



# MANUAL OF BACTERIOLOGY





# MANUAL OF BACTERIOLOGY.



## CHAPTER I.

### GENERAL MORPHOLOGY AND BIOLOGY.

**Introductory.**—At the bottom of the scale of living things there exists a group of organisms to which the name of bacteria is usually applied. These are apparently of very simple structure and may be subdivided into two sub-groups, a lower and simpler and a higher and better developed.

The *lower forms* are the more numerous, and consist of minute unicellular masses of protoplasm devoid of chlorophyll, which multiply by simple fission. Some are motile, others non-motile. Their minuteness may be judged of by the fact that in one direction at least they usually do not measure more than  $1\ \mu$  ( $\frac{1}{25000}$  inch). These forms can be classified according to their shapes into three main groups—(1) A group in which the shape is globular. The members of this are called *cocci*. (2) A group in which the shape is that of a straight rod—the proportion of the length to the breadth of the rod varying greatly among the different members. These are called *bacilli*. (3) A group in which the shape is that of a curved or spiral rod. These are called *spirilla*. The full description of the characters of these groups will be more conveniently taken later (p. 11). In some cases, especially among the bacilli, there may occur under certain circumstances changes in the protoplasm whereby a resting stage or spore is formed.

The *higher forms* show advance on the lower along two lines. (1) On the one hand they consist of filaments made up of simple elements such as occur in the lower forms. These

individuals may easily take place, especially in a fluid medium in which they may float entirely free from one another. Many of the higher bacteria possess a sheath which has a much more definite structure than is found among the lower forms. It resists external influences, possesses elasticity, and serves to bind the elements of the organism together.

**Reproduction among the Lower Bacteria.**—When a bacterial cell is placed in favourable surroundings it multiplies; as has been said, this, in the great majority of cases, takes place by simple fission. In the process a constriction appears in the middle and a transverse unstained line develops across the protoplasm at that point. The process goes on till two individuals can be recognised, which may remain for a time attached to one another, or become separate, according to the character of the envelope, as already explained. In most bacteria growth and multiplication go on with great rapidity. A bacterium may reach maturity and divide in from twenty minutes to half an hour. If division takes place only every hour, from one individual after twenty-four hours 17,000,000 similar individuals will be produced. As shown by the results of artificial cultivation, others, such as the tubercle bacillus, multiply much more slowly. Sometimes division proceeds so rapidly that the young individuals do not reach the adult size before multiplication again occurs. This may give rise to anomalous appearances. When bacteria are placed in unfavourable conditions as regards food, etc., growth and multiplication take place with difficulty. In the great majority of cases this is evidenced by changes in the appearance of the protoplasm. Instead of its maintaining the regularity of shape seen in healthy bacteria, various aberrant appearances are presented. This occurs especially in the rod-shaped varieties, where flask-shaped or dumb-bell-shaped individuals may be seen. The regularity in structure and size is quite lost. The appearance of the protoplasm also is often altered. Instead of, as formerly, staining well, it does not stain readily, and may have a uniformly pale, homogeneous appearance, while in an old culture only a small proportion of the bacteria may stain at all. Sometimes, on the other hand, a degenerated bacterium contains intensely stained granules or globules which may be of large size. Such aberrant and degenerate appearances are referred to as *involution forms*. That these forms really betoken degenerative changes is shown by the fact that, on their being again transferred to favourable conditions, only slight growth at first takes place. Many individuals have undoubtedly died, and the remainder which

live and develop into typical forms may sometimes have lost some of their properties.

**Reproduction among the Higher Bacteria.**—Most of the higher bacteria consist of thread-like structures more or less septate and often surrounded by a sheath. The organism is frequently attached at one end to some object or to another individual. It grows to a certain length and then at the free end certain cells called gonidia are cast off from which new individuals are formed. These gonidia may be formed by a division taking place in the terminal element of the filament such as has occurred in the growth of the latter. In some cases, however, division takes place in three dimensions of space. The gonidia have a free existence for a certain time before becoming attached, and in this stage are sometimes motile. They are usually rod-like in shape, sometimes pyriform. They do not possess any special powers of resistance.

**Spore Formation.**—In certain species of the lower bacteria, under certain circumstances, changes take place in the protoplasm which result in the formation of bodies called spores, to which the vital activities of the original bacteria are transferred. Spore formation occurs chiefly among the bacilli and in some spirilla. Its commencement in a bacterium is indicated by the appearance in the protoplasm of a minute highly refractile granule unstained by the ordinary methods. This increases in size, and assumes a round, oval, or short rod-shaped form, always shorter but often broader than the original bacterium. In the process of spore formation the rest of the bacterial protoplasm may remain unchanged in appearance and staining power for a considerable time (*e.g.* *b. tetani*), or, on the other hand, it may soon lose its power of staining and ultimately disappear, leaving the spore in the remains of the envelope (*e.g.* *b. anthracis*). This method of spore formation is called *endogenous*. Bacterial spores are always non-motile. The spore may appear in the centre of the bacterium, or it may be at one extremity, or a short distance from one extremity (Fig. 1, No. 11). In structure the spore consists of a mass of protoplasm surrounded by a dense membrane. This can be demonstrated by methods which will be described, the underlying principle of which is the prolonged application of a powerful stain. The membrane is supposed to confer on the spore its characteristic feature, namely, great capacity of resistance to external influences such as heat or noxious chemicals. Koch, for instance, in one series of experiments, found that while the bacillus anthracis in the unspored form was killed by a two minutes' exposure to 1 per cent carbolic acid, spores of the same organism resisted an exposure of from one to fifteen days.

When a spore is placed in suitable surroundings for growth

## 6 GENERAL MORPHOLOGY AND BIOLOGY

it again assumes the original bacillary or spiral form. The capsule dehisces either longitudinally, or terminally, or transversely. In the last case the dehiscence may be partial, and the new individual may remain for a time attached by its ends to the hinged spore-case, or the dehiscence may be complete and the bacillus grow with a cap at each end consisting of half the spore-case. Sometimes the spore-case does not dehisce, but is simply absorbed by the developing bacterium.

It is important to note that in the bacteria spore formation is rarely, if ever, to be considered as a method of multiplication. In at least the great majority of cases only one spore is formed from one bacterium, and only one bacterium in the first instance from one spore. Sporulation is to be looked upon as a *resting stage* of a bacterium, and is to be contrasted with the stage when active multiplication takes place. The latter is usually referred to as the *vegetative stage* of the bacterium. Regarding the signification of spore formation in bacteria there has been some difference of opinion. According to one view it may be regarded as representing the highest stage in the vital activity of a bacterium. There is thus an alternation between the vegetative and spore stage, the occurrence of the latter being necessary to the maintenance of the species in its greatest vitality. Such a rejuvenescence, as it were, through sporulation, is known in many algæ. In support of this view there are certain facts. In many cases, for instance, spore formation only occurs at temperatures specially favourable for growth and multiplication. There is often a temperature below which, while vegetative growth still takes place, sporulation will not occur; and in the case of *b. anthracis*, if the organism be kept at a temperature above the limit at which it grows best, not only are no spores formed, but the species may lose the power of sporulation. Furthermore, in the case of bacteria preferring the presence of oxygen for their growth, an abundant supply of this gas may favour sporulation. It is probable that even among bacteria preferring the absence of oxygen for vegetative growth, the presence of this gas favours sporulation. Most bacteriologists are, however, of opinion that when a bacterium forms a spore, it only does so when its surroundings, especially its food supply, become unfavourable for vegetative growth; it then remains in this condition until it is placed in more suitable surroundings. Such an occurrence would be analogous to what takes place under similar conditions in many of the protozoa. Often sporulation can be prevented from taking place for an indefinite time if a bacterium is constantly supplied with fresh food (the

other conditions of life being equal). The presence of substances excreted by the bacteria themselves plays, however, a more important part in making the surroundings unfavourable than the mere exhaustion of the food supply. A living spore will always develop into a vegetative form if placed in a fresh food supply. With regard to the rapid formation of spores when the conditions are favourable for vegetative growth, it must be borne in mind that in such circumstances the conditions may really very quickly become unfavourable for a continuance of growth, since not only will the food supply around the growing bacteria be rapidly exhausted, but the excretion of effete and inimical matters will be all the more rapid.

We must note that the usually applied tests of a body developed within a bacterium being a spore are (1) its staining reaction, namely, resistance to ordinary staining fluids, but capacity of being stained by the special methods devised for the purpose (*vide* p. 102); (2) the fact that the bacterium containing the spore has higher powers of resistance against inimical conditions than a vegetative form. It is important to bear these tests in mind, as in some of the smaller bacteria especially, it is very difficult to say whether they spore or not. There may appear in such organisms small unstained spots the significance of which it is very difficult to determine.

**The Question of Arthrosporous Bacteria.**—It is stated by Hueppe that among certain organisms, *e.g.* some streptococci, certain individuals may, without endogenous sporulation, take on a resting stage. These become swollen, stain well with ordinary stains, and they are stated to have higher power of resistance than the other forms; further, when vegetative life again occurs it is from them that multiplication is said to take place. From the fact that there is no new formation within the protoplasm, but that it is the whole of the latter which participates in the change, these individuals have been called *arthrospores*. The existence of such special individuals amongst the lower bacteria is extremely problematical. They have no distinct capsule, and they present no special staining reactions, nor any microscopic features by which they can be certainly recognised, while their alleged increased powers of resistance are very doubtful. All the phenomena noted can be explained by the undoubted fact that in an ordinary growth there is very great variation among the individual organisms in their powers of resistance to external conditions.

**Motility.**—As has been stated, many bacteria are motile. Motility can be studied by means of hanging-drop preparations (*vide* p. 63). The movements are of a darting, rolling, or vibratile character. The degree of motility depends on the species, the temperature, the age of the growth, and on the medium in which the bacteria are growing. Sometimes the movements are most active just after the cell has multiplied,

## 8 GENERAL MORPHOLOGY AND BIOLOGY

sometimes it goes on all through the life of the bacterium, sometimes it ceases when sporulation is about to occur. Motility is associated with the possession of fine wavy thread-like appendages called flagella, which for their demonstration require the application of special staining methods (*vide* Fig. 1, No. 12; and Fig. 112). They have been shown to occur in many bacilli and spirilla, but only in a few species of cocci. They vary in length, but may be several times the length of the bacterium, and may be at one or both extremities or all round. When terminal they may occur singly or there may be several. The nature of these flagella has been much disputed. Some have held that, unlike what occurs in many algæ, they are not actual prolongations of the bacterial protoplasm, but merely appendages of the envelope, and have doubted whether they are really organs of locomotion. There is now, however, little doubt that they belong to the protoplasm. By appropriate means the central parts of the latter can be made to shrink away from the peripheral (*vide infra*, "plasmolysis"). In such a case movement goes on as before, and in stained preparations the flagella can be seen to be attached to the peripheral zone. It is to be noted that flagella have never been demonstrated in non-motile bacteria, while, on the other hand, they have been observed in nearly all motile forms. There is little doubt, however, that all cases of motility among the bacteria are not dependent on the possession of flagella, for in some of the special spiral forms, and in most of the higher bacteria, motility is probably due to contractility of the protoplasm itself.

**The Minuter Structure of the Bacterial Protoplasm.**—Many attempts have been made to obtain deeper information as to the structure of the bacterial cell, and especially as to its behaviour in division. These have largely turned on the interpretation to be put on certain appearances which have been observed. These appearances are of two kinds. First, under certain circumstances irregular deeply-stained granules are observed in the protoplasm, often, when they occur in a bacillus, giving the latter the appearance of a short chain of cocci. They are often called metachromatic granules (*vide* Fig. 1, No. 16) from the fact that by appropriate procedure they can be stained with one dye, and the protoplasm in which they lie with another; sometimes, when a single stain is used, such as methylene blue, they assume a slightly different tint from the protoplasm.

For the demonstration of the metachromatic granules two methods have been advanced. Ernst recommends that a few drops of Löffler's methylene blue (*vide* p. 98) be placed on a cover-glass preparation and the latter passed backwards and forwards over a Bunsen flame for half a minute after steam begins to rise. The preparation is then washed in water and counter-stained for one to two minutes in watery Bismarck-brown. The granules are here stained blue, the protoplasm brown.

Neisser stains a similar preparation in warm carbol-fuchsin, washes with 1 per cent sulphuric acid, and counter-stains with Löffler's blue. Here the granules are magenta, the protoplasm blue. The general character of the granules thus is that they retain the first stain more intensely than the rest of the protoplasm does.

A second appearance which can sometimes be seen in specimens stained in ordinary ways is the occurrence of a concentration of the protoplasm at each end of a bacterium, indicated by these parts being deeply stained. These deeply stained parts are sometimes called polar granules (*vide* Fig. 1, No. 16, the bacillus most to the right), (German, Polkörnchen or Polkörner).

With regard to the significance that is to be attached to such appearances, much depends on whether they are constantly present under all circumstances, or only occasionally, when the organism is grown in special media or under special growth conditions. Some bacteria, however stained, show evidence of having the protoplasm somewhat granular, *e.g.* the diphtheria bacillus. In other cases this granular condition is only seen when the organism has been grown under bad conditions, or where the food supply is becoming exhausted. Some have thought that the appearances might be due to a process allied to mitosis and might signify approaching division, but of this there is no evidence.

In perfectly healthy and young bacteria, appearances of granule formation and of vacuolation may be accidentally produced by physical means in the occurrence of what is known as *plasmolysis*. To speak generally, when a mass of protoplasm surrounded by a fairly firm envelope of a colloidal nature is placed in a solution containing salts in greater concentration than that in which it has previously been living, then by a process of osmosis the water held in the protoplasm passes out through the membrane, and, the protoplasm retracting from the latter, the appearance of vacuolation is presented. Now in making a dried film for the microscopic examination of bacteria the conditions necessary for the occurrence of this process may be produced, and the appearances of vacuolation and, in certain cases, of Polkörner may thus be brought about. Plasmolysis in bacteria has been extensively investigated,<sup>1</sup> and has been found to occur in some species more readily than in others. Furthermore it is often most readily observed in old or otherwise enfeebled cultures.

Bütschli, from a study of some large sulphur-containing forms, concludes that the greater part of the bacterial cell may correspond to a nucleus, and that this is surrounded by a thin layer of protoplasm which in the smaller bacteria escapes notice, unless when, as in the bacilli, it can be made out at the ends of the cells. Fischer, it may be said, looks on the appearances seen in Bütschli's preparations as due to plasmolysis.

**The Chemical Composition of Bacteria.**—In the bodies of bacteria many definite substances occur. Some bacteria have been described as containing chlorophyll, but these are properly to be classed with the schizophyceæ. Sulphur is found in some of the higher forms, and starch granules are also described as occurring. Many species of bacteria, when growing in masses,

<sup>1</sup> Consult Fischer, "Untersuchungen über Bakterien," Berlin, 1894; "Ueber den Bau der Cyanophyceen und Bakterien," Jena, 1897.



are brilliantly coloured, though few bacteria associated with the production of disease give rise to pigments. In some of the organisms classed as bacteria a pigment named bacterio-purpurin has been observed in the protoplasm, and similar intracellular pigments probably occur in some of the larger forms of the lower bacteria and may occur in the smaller; but it is usually impossible to determine whether the pigment occurs inside or outside the protoplasm. In many cases, for the free production of pigment abundant oxygen supply is necessary; but sometimes, as in the case of *spirillum rubrum*, the pigment is best formed in the absence of oxygen. Sometimes the faculty of forming it may be lost by an organism for a time, if not permanently, by the conditions of its growth being altered. Thus, for example, if the *b. pyocyaneus* be exposed to the temperature of  $42^{\circ}\text{C}$ . for a certain time, it loses its power of producing its bluish pigment. Pigments formed by bacteria often diffuse out into, and colour, the medium for a considerable distance around.

Comparatively little is known of the nature of bacterial pigments. Zopf, however, has found that many of them belong to a group of colouring matters which occur widely in the vegetable and animal kingdoms, viz. the lipochromes. These lipochromes, which get their name from the colouring matter of animal fat, include the colouring matter in the petals of *Ranunculaceæ*, the yellow pigments of serum and of the yolks of eggs, and many bacterial pigments. The lipochromes are characterised by their solubility in chloroform, alcohol, ether, and petroleum, and by their giving indigo-blue crystals with strong sulphuric acid, and a green colour with iodine dissolved in potassium iodide. Though crystalline compounds of these have been obtained, their chemical constitution is entirely unknown and even their percentage composition is disputed.

Some observations have been made on the chemical structure of bacterial protoplasm. Nencki isolated from the bodies of certain putrefactive bacteria proteid bodies which, according to Ruppel, appear to have been allied to peptone, and which differed from nucleo-proteids in not containing phosphorus, but many of the proteids isolated by other chemists have been allied in their nature to the protoplasm of the nuclei of cells. Buchner in certain researches obtained bodies of this nature allied to the vegetable caseins, and he adduces evidence to show that it is to these that the characteristic staining properties are due. Various observers have isolated similar phosphorus-containing proteids from different bacteria. Besides proteids, however, substances of a different nature have been isolated. Thus cellulose, fatty material, chitin, wax-like bodies, and other substances have been observed. There are also found

various mineral salts, especially those of sodium, potassium, and magnesium. The amount of different constituents varies according to the age of the culture and the medium used for growth, and certainly great variation takes place in the composition of different species.

**The Classification of Bacteria.**—There have been numerous schemes set forth for the classification of bacteria, the fundamental principle running through all of which has been the recognition of the two sub-groups and the type forms mentioned in the opening paragraph above. In the attempts to still further subdivide the group, scarcely two systematists are agreed as to the characters on which sub-classes are to be based. Our present knowledge of the essential morphology and relations of bacteria is as yet too limited for a really natural classification to be attempted. To prepare for the elaboration of the latter, Marshall Ward suggested that in every species there should be studied the habitat, best food supply, condition as to gaseous environment, range of growth, temperature, morphology, life history, special properties and pathogenicity.

We must thus be content with a provisional and incomplete classification. We have said that the division into lower and higher bacteria is recognised by all, though, as in every other classification, there occur transitional forms. In subdividing the bacteria further, the forms they assume constitute at present the only practicable basis of classification. The lower bacteria thus naturally fall into the three groups mentioned, the cocci, bacilli, and spirilla, though the higher are more difficult to deal with. Subsidiary, though important, points in still further subdivision are the planes in which fission takes place and the presence or absence of spores. The recognition of actual species is often a matter of great difficulty. The points to be observed in this will be discussed later (p. 115).

**I. The Lower Bacteria.**<sup>1</sup>—These, as we have seen\*, are minute unicellular masses of protoplasm surrounded by an envelope, the total vital capacities of a species being represented in every cell. They present three distinct type forms, the coccus, the bacillus, and the spirillum; endogenous sporulation may occur. They may also be motile.

1. *The Cocci.*—In this group the cells range in different species from  $\cdot 5 \mu$  to  $2 \mu$  in diameter, but most measure about  $1 \mu$ . Before division they may increase in size in all directions. The species are usually classified according to the method of division.

<sup>1</sup> For the illustration of this and the succeeding systematic paragraphs, *vide* Fig. 1.

If the cells divide only in one axis, and through the consistency of their envelopes remain attached, then a chain of cocci will be formed. A species in which this occurs is known as a *streptococcus*. If division takes place irregularly the resultant mass may be compared to a bunch of grapes, and the species is often called a *staphylococcus*. Division may take place in two axes at right angles to one another, in which case cocci adherent to each other in packets of four (called *tetrads*) or sixteen may be found, the former number being the more frequent. To all these forms the word *micrococcus* is often generally applied. The individuals in a growth of micrococci often show a tendency to remain united in twos. These are spoken of as *diplococci*, but this is not a distinctive character, since every coccus as a result of division becomes a diplococcus, though in some species the tendency to remain in pairs is well marked. The adhesion of cocci to one another depends on the character of the capsule. Often this has a well-marked outer limit (*micrococcus tetragenus*), sometimes it is of great extent, its diameter being many times that of the coccus (*streptococcus mesenteriodes*). It is especially among the streptococci and staphylococci that the phenomenon of the formation of arthrospores is said to occur. In none of the cocci have endogenous spores been certainly observed. The number of species of the streptococci and staphylococci probably exceeds 150. Usually included in this group are coccus-like organisms which divide in three axes at right angles to one another. These are usually referred to as *sarcinae*. If the cells are lying single they are round, but usually they are seen in cubes of eight with the sides which are in contact slightly flattened. Large numbers of such cubes may be lying together. The *sarcinae* are, as a rule, rather larger than the other members of the group. Most of the cocci are non-motile, but a few motile species possessing flagella have been described.

2. *Bacilli*.—These consist of long or short cylindrical cells, with rounded or sharply rectangular ends, usually not more than  $1\ \mu$  broad, but varying very greatly in length. They may be motile or non-motile. Where flagella occur, these may be distributed all round the organism, or only at one or both of the poles (*pseudomonas*). Several species are provided with sharply-marked capsules (b. *pneumoniae*). In many species endogenous sporulation occurs. The spores may be central or terminal, round, oval, or spindle-shaped.

Great confusion in nomenclature has arisen in this group in consequence of the different artificial meanings assigned to the essentially synonymous terms bacterium and bacillus. Migula, for instance, applies

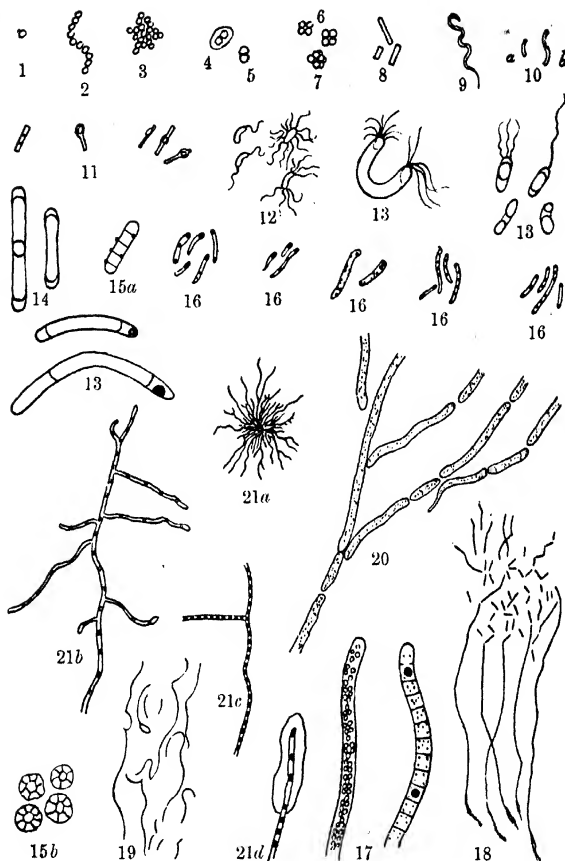


FIG. 1.—1. Coccus. 2. Streptococcus. 3. Staphylococcus. 4. Capsulated diplococcus. 5. "Biscuit"-shaped coccus. 6. Tetrads. 7. Sarcina form. 8. Types of bacilli (1-8 are diagrammatic). 9. Non-septate spirillum  $\times 1000$ . 10. Ordinary spirillum—(a) comma-shaped element; (b) formation of spiral by comma-shaped elements  $\times 1000$ . 11. Types of spore formation. 12. Flagellated bacteria. 13. Changes in bacteria produced by plasmolysis (after Fischer). 14. Bacilli with terminal protoplasm (Bütschli). 15. (a) Bacillus composed of five protoplasmic meshes; (b) protoplasmic network in micrococcus (Bütschli). 16. Bacteria containing metachromatic granules (Ernst, Neisser)—some contain polar granules. 17. *Beggiatoa alba*. Both filaments contain sulphur granules—one is septate. 18. *Thiothrix tenuis* (Winogradski). 19. *Leptothrix innominata* (Miller). 20. *Cladothrix dichotoma* (Zopf). 21. *Streptothrix actinomyces* (Boström), (a) colony under low power; (b) filament showing true branching; (c) filament containing coccus-like bodies; (d) filament with club at end.

## 14 GENERAL MORPHOLOGY AND BIOLOGY

the former term to non-motile species, the latter to the motile. Hueppe, on the other hand, calls those in which endogenous sporulation does not occur, bacteria, and those where it does, bacilli. In the ordinary terminology of systematic bacteriology the word bacterium has been almost dropped, and is reserved, as we have done, as a general term for the whole group. It is usual to call all the rod-shaped varieties bacilli.

3. *Spirilla*.—These consist of cylindrical cells more or less spiral or wavy. Of such there are two main types. In one there is a long non-septate, usually slender, wavy or spiral thread (Fig. 1, No. 9). In the other type the unit is a short curved rod (often referred to as of a "comma" shape). When two or more of the latter occur, as they often do, end to end with their curves alternating, then a wavy or spiral thread results. An example of this is the cholera microbe (Fig. 1, No. 10). This latter type is of much more frequent occurrence, and contains the more important species. Among the first group motility is often not associated, as far as is known, with the possession of flagella. The cells here apparently move by an undulating or screw-like contraction of the protoplasm. Most of the motile spirilla, however, possess flagella. Of the latter there may be one or two, or a bunch containing as many as twenty, at one or both poles. Division takes place as among the bacilli, and in some species endogenous sporulation has been observed.

Three terms are used in dividing this group, to which different authors have given different meanings. These terms are spirillum, spirochæte, vibrio. Migula makes "vibrio" synonymous with "microspira," which he applies to members of the group which possess only one or two polar flagella; "spirillum" he applies to similar species which have bunches of polar flagella, while "spirochæte" is reserved for the long unflagellated spiral cells. Hueppe applies the term "spirochæte" to forms without endospores, "vibrio" to those with endospores in which during sporulation the organism changes its form, and "spirillum" to the latter when no change of form takes place in sporulation. Flugge, another systematist, applies "spirochæte" and "spirillum" indiscriminately to any wavy or corkscrew form, and "vibrio" to forms where the undulations are not so well marked. It is thus necessary, in denominating such a bacterium by a specific name, to give the authority from whom the name is taken.

Quite recently great doubt has arisen as to whether many of the non-septate spirillary forms are to be looked on as bacteria at all,—the view being taken that in, it may be, many cases they represent a stage in the life history of what are really protozoa of the nature of trypanosomes. The ultimate classification of the spirilla must thus be left an open question.

**II. The Higher Bacteria.**—These show advance on the lower in consisting of definite filaments branched or unbranched. In

most cases the filaments at more or less regular intervals are cut by septa into short rod-shaped or curved elements. Such elements are more or less interdependent on one another, and special staining methods are often necessary to demonstrate the septa which demarcate the individuals of a filament. There is further often a definite membrane or sheath common to all the elements in a filament. Not only, however, is there this close organic relationship between the elements of the higher bacteria, but there is also interdependence of function; for example, one end of a filament is frequently concerned merely in attaching the organism to some other object. The greatest advance, however, consists in the setting apart among most of the higher bacteria of the free terminations of the filaments for the production of new individuals, as has been described (p. 2). There are various classes under which the species of the higher bacteria are grouped; but our knowledge of them is still somewhat limited, as many of the members have not yet been artificially cultivated. The *beggiatoa* group consists of free swimming forms, motile by undulating contractions of their protoplasm. For the demonstration of the rod-like elements of the filaments special staining is necessary. The filaments have no special sheath, and the protoplasm contains sulphur granules. The method of reproduction is doubtful. The *thiothrix* group resembles the last in structure, and the protoplasm also contains sulphur granules; but the filaments are attached at one end, and at the other form gonidia. The *leptothrix* group resembles closely the *thiothrix* group, but the protoplasm does not contain sulphur granules. In the *cladothrix* group there is the appearance of branching, which, however, is of a false kind. What happens is that a terminal cell divides, and on dividing again, it pushes the product of its first division to one side. There are thus two terminal cells lying side by side, and as each goes on dividing, the appearance of branching is given. Here, again, there is gonidium formation; and while the parent organism is in some of its elements motile, the gonidia move by means of flagella. The highest development is in the *streptothrix* group, to which belongs the *streptothrix actinomyces*, or the *actinomyces bovis*, and several other important pathogenic agents. Here the organism consists of a felted mass of non-septate filaments, in which true dichotomous branching occurs. Under certain circumstances threads grow out, and produce chains of coccus-like bodies from which new individuals can be reproduced. Such bodies are often referred to as spores, but they have not the same staining reactions nor resisting powers of so high a degree as ordinary bacterial

spores. Sometimes too the protoplasm of the filaments breaks up into bacillus-like elements, which may also have the capacity of originating new individuals. In the streptothrix actinomyces there may appear a club-shaped swelling of the membrane at the end of the filament, which has by some been looked on as an organ of fructification, but which is most probably a product of a degenerative change. The streptothrix group, though its morphology and relationships are much disputed, may be looked on as a link between the bacteria on the one hand, and the lower fungi on the other. Like the latter, the streptothrix forms show the felted mass of non-septate branching filaments, which is usually called a mycelium. On the other hand, the breaking up of the protoplasm of the streptothrix into coccus- and bacillus-like forms, links it to the other bacteria.

#### GENERAL BIOLOGY OF THE BACTERIA.

There are five prime factors which must be considered in the growth of bacteria, namely, food supply, moisture, relation to gaseous environment, temperature, and light.

**Food Supply.**—The bacteria are chiefly found living on the complicated organic substances which form the bodies of dead plants and animals, or which are excreted by the latter while they are yet alive. Seeing that, as a general rule, many bacteria grow side by side, the food supply of any particular variety is, relatively to it, altered by the growth of the other varieties present. It is thus impossible to imitate the complexity of the natural food environment of any species. The artificial media used in bacteriological work may therefore be poor substitutes for the natural supply. In certain cases, however, the conditions under which we grow cultures may be better than the natural conditions. For while one of two species of bacteria growing side by side may favour the growth of the other, it may also in certain cases hinder it, and, therefore, when the latter is grown alone it may grow better. Most bacteria seem to produce excretions which are unfavourable to their own vitality, for, when a species is sown on a mass of artificial food medium, it does not in the great majority of cases go on growing till the food supply is exhausted, but soon ceases to grow. Effete products diffuse out into the medium and prevent growth. Such diffusion may be seen when the organism produces pigment, *e.g.* *b. pyocyaneus* growing on gelatin. In supplying artificial food for bacterial growth, the general principle ought to be to imitate as nearly as possible the natural surround-

ings, though it is found that there exists a considerable adaptability\* among organisms. With the pathogenic varieties it is usually found expedient to use media derived from the fluids of the animal body, and in cases where bacteria growing on plants are being studied, infusions of the plants on which they grow are frequently used. Some bacteria can exist on inorganic food, but most require organic material to be supplied. Of the latter, some require for their proper nourishment proteid to be present, while others can derive their nitrogen from such a non-proteid as asparagin. All bacteria require nitrogen to be present in some form, and many require to derive their carbon from carbohydrates. Mineral salts, especially sulphates, chlorides, and phosphates, and also salts of iron are necessary. Occasionally special substances are needed to support life. Thus some species, in the protoplasm of which sulphur granules occur, require sulphuretted hydrogen to be present. In nature the latter is usually provided by the growth of other bacteria. When the food supply of a bacterium fails, it degenerates and dies. The proof of death lies in the fact that when it is transferred to fresh and good food supply it does not multiply. If the bacterium spores, it may then survive the want of food for a very long time. It may here be stated that the reaction of the food medium is a matter of great importance. Most bacteria prefer a slightly alkaline medium, and some, *e.g.* the cholera spirillum, will not grow in the presence of the smallest amount of free acid.

**Moisture.**—The presence of water is necessary for the continued growth of all bacteria. The amount of drying which bacteria in the vegetative stage will resist varies very much in different species. Thus the cholera spirillum is killed by two or three hours' drying, while the staphylococcus pyogenes aureus will survive ten days' drying, and the bacillus diphtheriæ still more. In the case of spores the periods are much longer. Anthrax spores will survive drying for several years, but here again moisture enables them to resist longer than when they are quite dry. When organisms have been subjected to such hostile influences, even though they survive, it by no means follows that they retain all their vital properties.

**Relation to Gaseous Environment.**—The relation of bacteria to the oxygen of the air is such an important factor in the life of bacteria that it enables a biological division to be made among them. Some bacteria will only live and grow when oxygen is present. To these the title of *obligatory aerobes* is given. Other bacteria will only grow when no oxygen is present. These are



called *obligatory anaerobes*. In still other bacteria the presence or absence of oxygen is a matter of indifference. This group might theoretically be divided into those which are preferably aerobes, but can be anaerobes, and those which are preferably anaerobes, but can be aerobes. As a matter of fact such differences are manifested to a slight degree, but all such organisms are usually grouped as *facultative anaerobes*, i.e. preferably aerobic but capable of existing without oxygen. Examples of obligatory aerobes are *b. proteus vulgaris*, *b. subtilis*; of obligatory anaerobes, *b. tetani*, *b. oedematis maligni*, while the great majority of pathogenic bacteria are facultative anaerobes. With regard to anaerobes, hydrogen and nitrogen are indifferent gases. Many anaerobes, however, do not flourish well in an atmosphere of carbon dioxide. Very few experiments have been made to investigate the action on bacteria of gas under pressure. A great pressure of carbon dioxide is said to make the *b. anthracis* lose its power of sporing, but it seems to have no effect on its vitality or on that of the *b. typhosus*. With the *bacillus pyocyaneus*, however, it is said to destroy life.

**Temperature.**—For every species of bacterium there is a temperature at which it grows best. This is called the “optimum temperature.” There is also in each case a maximum temperature above which growth does not take place, and a minimum temperature below which growth does not take place. As a general rule the optimum temperature is about the temperature of the natural habitat of the organism. For organisms taking part in the ordinary processes of putrefaction the temperature of warm summer weather ( $20^{\circ}$  to  $24^{\circ}$  C.) may be taken as the average optimum, while for organisms normally inhabiting animal tissues  $35^{\circ}$  to  $39^{\circ}$  C is a fair average. The lowest limit of ordinary growth is from  $12^{\circ}$  to  $14^{\circ}$  C., and the upper is from  $42^{\circ}$  to  $44^{\circ}$  C. In exceptional cases growth may take place as low as  $5^{\circ}$  C., and as high as  $70^{\circ}$  C. Some organisms which grow best at a temperature of from  $60^{\circ}$  to  $70^{\circ}$  C. have been isolated from dung, the intestinal tract, etc. These have been called *thermophilic* bacteria. It is to be noted that while growth does not take place below or above a certain limit it by no means follows that death takes place outside such limits. Organisms can resist cooling below their minimum or heating beyond their maximum without being killed. Their vital activity is merely paralysed. Especially is this true of the effect of cold on bacteria. The results of different observers vary; but if we take as an example the cholera vibrio, Koch found that while the minimum temperature of growth was  $16^{\circ}$  C., a culture might

be cooled to  $-32^{\circ}\text{C}$ . without being killed. With regard to the upper limit, few ordinary organisms in a spore-free condition will survive a temperature of  $57^{\circ}\text{C}$ ., if long enough applied. Many organisms lose some of their properties when grown at unnatural temperatures. Thus many pathogenic organisms lose their virulence if grown above their optimum temperature, and some chromogenic forms, most of which prefer rather low temperatures, lose their capacity of producing pigment, *e.g.* *spirillum rubrum*.

**Effect of Light.**—Of recent years much attention has been paid to this factor in the life of bacteria. Direct sunlight is found to have a very inimical effect. It has been found that an exposure of dry anthrax spores for one and a half hours to sunlight kills them. When they are moist, a much longer exposure is necessary. Typhoid bacilli are killed in about one and a half hours, and similar results have been obtained with many other organisms. In such experiments the thickness of the medium surrounding the growth is an important point. Death takes place more readily if the medium is scanty or if the organisms are suspended in water. Any fallacy which might arise from the effect of the heat rays of the sun has been excluded, though light plus heat is more fatal than light alone. In direct sunlight it is chiefly the green, violet, and, it may be, the ultra-violet rays which are fatal. Diffuse daylight has also a bad effect upon bacteria, though it takes a much longer exposure to do serious harm. A powerful electric light is as fatal as sunlight. Here, as with other factors, the results vary very much with the species under observation, and a distinction must be drawn between a mere cessation of growth and the condition of actual death. Some bacteria especially occurring on the dead bodies of fresh fish are phosphorescent.

**Conditions affecting the Movements of Bacteria.**—In some cases differences are observed in the behaviour of motile bacteria, contemporaneous with changes in their life history. Thus, in the case of *bacillus subtilis*, movement ceases when sporulation is about to take place. On the other hand, in the *bacillus* of symptomatic anthrax, movement continues while sporulation is progressing. Under ordinary circumstances motile bacteria appear not to be constantly moving but occasionally to rest. In every case the movements become more active if the temperature be raised. Most interest, however, attaches to the fact that bacilli may be attracted to certain substances and repelled by others. Schenk, for instance, observed that motile bacteria were attracted to a warm point in a way which did not

occur when the bacteria were dead and therefore only subject to physical conditions. Most important observations have been made on the attraction and repulsion exercised on bacteria by chemical agents, which have been denominated respectively *positive* and *negative chemiotaxis*. Pfeffer investigated this subject in many lowly organisms, including bacterium termo and spirillum undula. The method was to fill with the agent a fine capillary tube, closed at one end, to introduce this into a drop of fluid containing the bacteria under a cover-glass, and to watch the effect through the microscope. The general result was to indicate that motile bacteria may be either attracted or repelled by the fluid in the tube. The effect of a given fluid differs in different organisms, and a fluid chemiotactic for one organism may not act on another. Degree of concentration is important, but the nature of the fluid is more so. Of inorganic bodies salts of potassium are the most powerfully attracting bodies, and in comparing organic bodies the important factor is the molecular constitution. These observations have been confirmed by Ali-Cohen, who found that while the vibrio of cholera and the typhoid bacillus were scarcely attracted by chloride of potassium, they were powerfully influenced by potato juice. Further, the filtered products of the growth of many bacteria have been found to have powerful chemiotactic properties. It is evident that all these observations have a most important bearing on the action of bacteria, though we do not yet know their true significance. Corresponding chemiotactic phenomena are shown also by certain animal cells, *e.g.* leucocytes, to which reference is made below.

**The Parts played by Bacteria in Nature.**—As has been said, the chief effect of bacterial action in nature is to break up into more simple combinations the complex molecules of the organic substances which form the bodies of plants and animals, or which are excreted by them. In some cases we know some of the stages of disintegration, but in most cases we know only general principles and sometimes only results. In the case of milk, for instance, we know that lactic acid is produced from the lactose by the action of the bacillus acidi lactici and of other bacteria, and that from urea ammonium carbonate is produced by the micrococcus ureae. That the very complicated process of putrefaction is due to bacteria is absolutely proved, for any organic substance can be preserved indefinitely from ordinary putrefaction by the adoption of some method of killing all bacteria present in it, as will be afterwards described. This statement, however, does not exclude the fact that molecular

changes take place spontaneously in the passing of the organic body from life to death. Many processes not usually referred to as putrefactive are also bacterial in their origin. The souring of milk, already referred to, the becoming rancid of butter, the ripening of cream and of cheese, are all due to bacteria.

A certain comparatively small number of bacteria have been proved to be the causal agents in some disease processes occurring in man, animals, and plants. This means that the fluids and tissues of living bodies are, under certain circumstances, a suitable pabulum for the bacteria involved. The effects of the action of these bacteria are analogous to those taking place in the action of the same or other bacteria on dead animal or vegetable matter. The complex organic molecules are broken up into simpler products. We shall study these processes more in detail later. Meantime we may note that the disease-producing effects of bacteria form the basis of another biological division of the group. Some bacteria are harmless to animals and plants, and apparently under no circumstances give rise to disease in either. These are known as saprophytes. They are normally engaged in breaking up dead animal and vegetable matter. Others normally live on or in the bodies of plants and animals and produce disease. These are known as parasitic bacteria. Sometimes an attempt is made to draw a hard and fast line between the *saprophytes* and the *parasites*, and obligatory saprophytes or parasites are spoken of. This is an erroneous distinction. Some bacteria which are normally saprophytes can produce pathogenic effects (*e.g.* bacillus oedematis maligni), and it is consistent with our knowledge that the best-known parasites may have been derived from saprophytes. On the other hand, the fact that most bacteria associated with disease processes, and proved to be the cause of the latter, can be grown in artificial media, shows that for a time at least such parasites can be saprophytic. As to how far such a saprophytic existence of disease-producing bacteria occurs in nature, we are in many instances still ignorant.

**The Methods of Bacterial Action.**—The processes which bodies undergo in being split up by bacteria depend, first, on the chemical nature of the bodies involved and, secondly, on the varieties of the bacteria which are acting. The destruction of albuminous bodies which is mostly involved in the wide and varied process of putrefaction can be undertaken by whole groups of different varieties of bacteria. The action of the latter on such substances is analogous to what takes place when albumins are subjected to ordinary gastric and intestinal digestion.

In these circumstances, therefore, the production of albumoses, peptones, etc., similar to those of ordinary digestion, can be recognised in putrefying solutions, though the process of destruction always goes further, and still simpler substances, *e.g.* indol, and, it may be, crystalline bodies of an alkaloidal nature, are the ultimate results. The process is an exceedingly complicated one when it takes place in nature, and different bacteria are probably concerned in the different stages. Many other bacteria, *e.g.* some pathogenic forms, though not concerned in ordinary putrefactive processes, have a similar digestive capacity. When carbohydrates are being split up, then various alcohols, ethers, and acids are produced. During bacterial growth there is not infrequently the abundant production of such gases as sulphuretted hydrogen, carbon dioxide, methane, etc. For an exact knowledge of the destructive capacities of any particular bacterium there must be an accurate chemical examination of its effects when it has been grown in artificial media the nature of which is known. The precise substances it is capable of forming can thus be found out. Many substances, however, are produced by bacteria, of the exact nature of which we are still ignorant, for example, the toxic bodies which play such an important part in the action of many pathogenic species.

Many of the actions of bacteria depend on the production by them of *ferments* of a very varied nature and complicated action. Thus the digestive action on albumins probably depends on the production of a peptic ferment analogous to that produced in the animal stomach. Ferments which invert sugar, which split sugars up into alcohols or acids, which coagulate casein, which split up urea into ammonium carbonate, also occur.

Such ferments may be diffused into the surrounding fluid, or be retained in the cells where they are formed. Sometimes the breaking down of the organic matter appears to take place within, or in the immediate proximity of, the bacteria, sometimes wherever the soluble ferments reach the organic substances. And in certain cases the ferments diffused out into the surrounding medium probably break down the constituents of the latter to some extent, and prepare them for a further, probably intracellular, disintegration. Thus in certain putrefactions of fibrin, if the process be allowed to go on naturally, the fibrin dissolves and ultimately great gaseous evolution of carbon dioxide and ammonia takes place, but if the bacteria, shortly after the process has begun, are killed or paralysed by chloroform, then only a peptonisation of the fibrin occurs, without the further splitting up and gaseous pro-

duction being observed. That a purely intracellular digestion may take place is illustrated by what has been shown to occur in the case of the micrococcus ureae, which from urea forms ammonium carbonate by adding water to the urea molecule. Here, if after the action has commenced, the bacteria are filtered off, no further production of ammonium carbonate takes place, which shows that no ferment has been dissolved out into the urine. If now the bodies of the bacteria be extracted with absolute alcohol or ether, which of course destroy their vitality, a substance is obtained of the nature of a ferment, which, when added to sterile urine, rapidly causes the production of ammonium carbonate. This ferment has evidently been contained within the bacterial cells.

In considering the effects of bacteria in nature it must be recognised that some species are capable of building up complex substances out of simple chemical compounds. Examples of these are found in the bacteria which in the soil make nitrogen more available for plant nutrition by converting ammonia into nitrites and nitrates. Winogradski, by using media containing non-nitrogenous salts of magnesium, potassium, and ammonium, and free of organic matter, has demonstrated the existence of forms which convert, by oxidation, ammonia into nitrites, and of other forms which convert these nitrites into nitrates. Both can derive their necessary carbon from alkaline carbonates. Other bacteria, or organisms allied to the bacteria, exist which can actually take up and combine into new compounds the free nitrogen of the air. These are found in the tubercles which develop on the rootlets of the leguminosae. Without such organisms the tubercles do not develop, and without the development of the tubercles the plants are poor and stunted. Bacteria thus play an important part in the enrichment and fertilisation of the soil.

**The Occurrence of Variability among Bacteria.**—The question of the division of the group of bacteria into definite species has given rise to much discussion among vegetable and animal morphologists, and at one time very divergent views were held. Some even thought that the same species might at one time give rise to one disease,—at another time to another. There is, however, now practical unanimity that bacteria show as distinct species as the other lower plants and animals, though, of course, the difficulty of defining the concept of a species is as great in them as it is in the latter. Still, we can say that among the bacteria we see exhibited (to use the words of De Bary) "the same periodically repeated course of development within certain empirically determined limits of variation" which justifies, among higher forms of life, a species to be recognised. What at first raised doubts as to the occurrence of species among the bacteria was the observation in certain cases of what is known as *pleomorphism*. By this is meant that one species may assume at different times different forms, *e.g.* appear as a coccus, a bacillus, or a leptothrix. Undoubtedly, many of the cases where this was alleged to have been observed occurred before the elaboration of the modern technique for the obtaining of pure cultures, but at the present day there are cases where evidence appears to exist

of the occurrence of pleomorphism. This is especially the case with certain bacilli, and it may lead to such forms being classed among the higher bacteria. Pleomorphism is, however, a rare condition, and with regard to the bacteria as a whole we may say that each variety tends to conform to a definite type of structure and function which is peculiar to it and to it alone. On the other hand, slight variations from such type can occur in each. The size may vary a little with the medium in which the organism is growing, and under certain similar conditions the adhesion of bacteria to each other may also vary. Thus cocci, which are ordinarily seen in short chains, may grow in long chains. The capacity to form spores may be altered, and such properties as the elaboration of certain ferments or of certain pigments may be impaired. Also the characters of the growths on various media may undergo variations. As has been remarked, variation as observed consists largely in a tendency in a bacterium to lose properties ordinarily possessed, and all attempts to transform one bacterium into an apparently closely allied variety (such as the *b. coli* into the *b. typhosus*) have failed. This of course does not preclude the possibility of one species having been originally derived from another or of both having descended from a common ancestor, but we can say that only variations of an unimportant order have been observed to take place, and here it must be remembered that in many cases we can have forty-eight or more generations under observation within twenty-four hours.

## CHAPTER II.

### METHODS OF CULTIVATION OF BACTERIA.

**Introductory.**—In order to study the characters of any species of bacterium it is necessary to have it growing apart from every other species. In the great majority of cases where bacteria occur in nature, this condition is not fulfilled. Only in the blood and tissues in some diseases do particular species occur singly and alone. We usually have, therefore, to remove a bacterium from its natural surroundings and grow it on an artificial food medium. When we have succeeded in separating it, and have got it to grow on a medium which suits it, we are said to have obtained a *pure culture*. The recognition of different species of bacteria depends, in fact, far more on the characters presented by pure cultures and their behaviour in different food media, than on microscopic examination. The latter in most cases only enables us to refer a given bacterium to its class. Again, in inquiring as to the possible possession of pathogenic properties by a bacterium, the obtaining of pure cultures is absolutely essential.

To obtain pure cultures, then, is the first requisite of bacteriological research. Now, as bacteria are practically omnipresent, we must first of all have means of destroying all extraneous organisms which may be present in the food media to be used in the vessels in which the food media are contained, and on all instruments which are to come in contact with our cultures. The technique of this destructive process is called sterilisation. We must therefore study the *methods of sterilisation*. The growth of bacteria in other than their natural surroundings involves further the *preparation of sterile artificial food media*, and when we have such media prepared we have still to look at the technique of the *separation of micro-organisms from mixtures of these, and the maintaining of pure cultures when the latter have been obtained*. We shall here find that different methods



## 26 METHODS OF CULTIVATION OF BACTERIA

are necessary according as we are dealing with *aerobes* or *anaerobes*. Each of these methods will be considered in turn.

### THE METHODS OF STERILISATION.

To exclude extraneous organisms, all food materials, glass vessels containing them, wires used in transferring bacteria from one culture medium to another, instruments used in making autopsies, etc., must be sterilised. These objects being so different, various methods are necessary, but underlying these methods is the general principle that all bacteria are destroyed by heat. The temperature necessary varies with different bacteria, and the vehicle of heat is also of great importance. The two vehicles employed are hot air and hot water or steam. The former is usually referred to as "dry heat," the latter as "moist heat." As showing the different effects of the two vehicles, Koch found, for instance, that the spores of *Bacillus anthracis*, which were killed by moist heat at  $100^{\circ}\text{C}$ ., in one hour, required three hours' dry heat at  $140^{\circ}\text{C}$ . to effect death. Both forms of heat may be applied at different temperatures—in the case of moist heat above  $100^{\circ}\text{C}$ ., a pressure higher than that of the atmosphere must of course be present.

#### A. *Sterilisation by Dry Heat.*

**A. (1) Red Heat or Dull Red Heat.**—Red heat is used for the sterilisation of the platinum needles which, it will be found, are so constantly in use. A dull heat is used for cauteries, the points of forceps, and may be used for the incidental sterilisation of small glass objects (cover-slips, slides, occasionally when necessary even test-tubes), care of course being taken not to melt the glass. The heat is obtained by an ordinary Bunsen burner.

**A. (2) Sterilisation by Dry Heat in a Hot-Air Chamber.**—The chamber (Fig. 2) consists of an outer and inner case of sheet iron. In the bottom of the outer there is a large hole. A Bunsen is lit beneath this, and thus plays on the bottom of the inner case, round all of the sides of which the hot air rises and escapes through holes in the top of the outer case. A thermometer passes down into the interior of the chamber, half-way up which its bulb should be situated. It is found as a matter of experience, that an exposure in such a chamber for

one hour to a temperature of  $170^{\circ}\text{C.}$ , is sufficient to kill all the organisms which usually pollute articles in a bacteriological laboratory, though circumstances might arise where this would be insufficient. This means of sterilisation is used for the glass flasks, test-tubes, plates, Petri's dishes, the use of which will be described. Such pieces of apparatus are thus obtained sterile and dry. It is advisable to put glass vessels into the chamber before heating it, and to allow them to stand in it after sterilisation till the temperature falls. Sudden heating or cooling is apt to cause glass to crack. The method is manifestly unsuitable for food media.

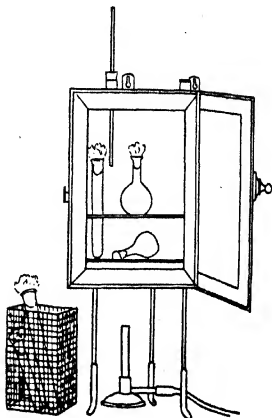


FIG. 2.—Hot-air steriliser.

### B. *Sterilisation by Moist Heat.*

**B. (1) By Boiling.**—The boiling of a liquid for five minutes is sufficient to kill ordinary germs if no spores be present, and this method is useful for sterilising distilled or tap water which may be required in various manipulations. It is best to sterilise knives and instruments used in autopsies by boiling in water to which a little sodium carbonate has been added to prevent rusting. Twenty minutes' boiling will here be sufficient. The boiling of any fluid at  $100^{\circ}\text{C.}$  for one and a half hours will ensure sterilisation under almost any circumstances.

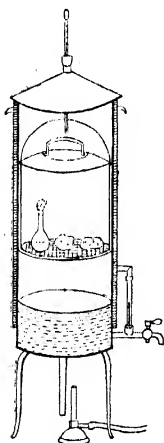


FIG. 3.—Koch's steam steriliser.

**B. (2) By Steam at  $100^{\circ}\text{C.}$** —This is by far the most useful means of sterilisation. It may be accomplished in an ordinary potato steamer placed on a kitchen pot. The apparatus ordinarily used is "Koch's steam steriliser" (Fig. 3). This consists of a tall metal cylinder on legs, provided with a lid, and covered externally by

some bad conductor of heat, such as felt or asbestos. A perforated tin diaphragm is fitted in the interior at a little distance above the bottom, and there is a tap at the bottom by which water may be supplied or withdrawn. If water to the depth of 3 inches be placed in the interior and heat applied, it will quickly boil, and the steam streaming up will surround any flask or other object standing on the diaphragm. Here no evaporation takes place from any medium as it is surrounded during sterilisation by an atmosphere saturated with water vapour. It is convenient to have the cylinder tall enough to hold a litre flask with a funnel 7 inches in diameter standing in its neck. The funnel may be supported by passing its tube through a second perforated diaphragm placed in the upper part of the steam chamber. With such a "Koch" in the laboratory a hot-water filter is not needed. As has been said, one and a half hour's steaming will sterilise any medium, but in the case of media containing gelatin such an exposure is not practicable, as with long boiling, gelatin tends to lose its physical property of solidification. The method adopted in this case is to *steam for a quarter of an hour on each of three succeeding days.*

This is a modification of what is known as "Tyndall's intermittent sterilisation." The fundamental principle of this method is that all bacteria in a non-spored form are killed by the temperature of boiling water, while if in a spored form they may not be thus killed. Thus by the sterilisation on the first day all the non-spored forms are destroyed—the spores remaining alive. During the twenty-four hours which intervene before the next heating, these spores, being in a favourable medium, are likely to assume the non-spored form. The next heating kills these. In case any may still not have changed their spored form, the process is repeated on a third day. Experience shows that usually the medium can now be kept indefinitely in a sterile condition.

Steam at 100° C. is therefore available for the sterilisation of all ordinary media. In using the Koch's steriliser, especially when a large bulk of medium is to be sterilised, it is best to put the media in while the apparatus is cold, in order to make certain that the whole of the food mass reaches the temperature of 100° C. The period of exposure is reckoned from the time boiling commences in the water in the steriliser. At any rate allowance must always be made for the time required to raise the temperature of the medium to that of the steam surrounding it.

If we wish to use such a substance as blood serum as a medium, the albumin would be coagulated by a temperature of 100° C. Therefore other means have to be adopted in this case.

**B. (3) Sterilisation by Steam at High Pressure.**—This is the most rapid and effective means of sterilisation. It is effected in an autoclave (Fig. 4). This is a gun-metal cylinder supported in a cylindrical sheet-iron case; its top is fastened down with screws and nuts and is furnished with a safety valve, pressure-gauge, and a hole for thermometer. As in the Koch's steriliser, the contents are supported on a perforated diaphragm. The source of heat is a large Bunsen beneath. The temperature employed is usually  $115^{\circ}\text{C.}$  or  $120^{\circ}\text{C.}$  To boil at  $115^{\circ}\text{C.}$ , water requires a pressure of about 23 lbs. to the square inch (*i.e.* 8 lbs. plus the 15 lbs. of ordinary atmospheric pressure). To boil at  $120^{\circ}\text{C.}$ , a pressure of about 30 lbs. (*i.e.* 15 lbs. plus the usual pressure) is necessary. In such an apparatus the desired temperature is maintained by adjusting the safety valve so as to blow off at the corresponding pressure. One exposure of media to such temperatures for a quarter of an hour is amply sufficient to kill all organisms or spores. Here, again, care must be taken when gelatin is to be sterilised. It must not be exposed to a temperature above  $105^{\circ}\text{C.}$ , and is best sterilised by the intermittent method. Certain precautions are necessary in using the autoclave. In all cases it is necessary to allow the apparatus to cool well below  $100^{\circ}\text{C.}$ , before opening it or allowing steam to blow off, otherwise there will be a sudden development of steam when the pressure is removed, and fluid media will be blown out of the flasks. Sometimes the instrument is not fitted with a thermometer. In this case care must be taken to expel all the air initially present, otherwise a mixture of air and steam being present, the pressure read off the gauge cannot be accepted as an indication of the temperature. Further, care must be taken to ensure the presence of a residuum of water when steam is fully up, otherwise the steam is superheated, and the pressure on the gauge again does not indicate the temperature correctly.

**B. (4) Sterilisation at Low Temperatures.**—Most organisms in a non-spored form are killed by a prolonged exposure to a temperature of  $57^{\circ}\text{C.}$  This fact has been taken advantage of for the sterilisation of blood serum, which will coagulate if exposed to a

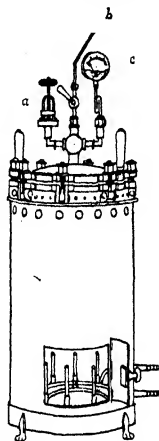


FIG. 4.—Autoclave.

- a. Safety-valve.
- b. Blow-off pipe.
- c. Gauge.

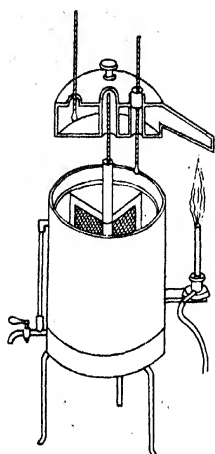


FIG. 5.—Steriliser for blood serum.

temperature above that point. Such a medium is sterilised on Tyndall's principle by exposing it for an hour at  $57^{\circ}$  C. for eight consecutive days, it being allowed to cool in the interval to the room temperature. The apparatus shown in Fig. 5 is a small hot-water jacket heated by a Bunsen placed beneath it, the temperature being controlled by a gas regulator. To ensure that the temperature all around shall be the same, the lid also is hollow and filled with water, and there is a special gas burner at the side to heat it. This is the form originally used, but serum sterilisers are now constructed in which the test-tubes are placed in the sloped position, and in which inspissation (*vide* p. 40) can afterwards be performed at a higher temperature.

### THE PREPARATION OF CULTURE MEDIA.

The general principle to be observed in the artificial culture of bacteria is that the medium used should approximate as closely as possible to that on which the bacterium grows naturally. In the case of pathogenic bacteria the medium therefore should resemble the juices of the body. The serum of the blood satisfies this condition and is often used, but its application is limited by the difficulties in its preparation and preservation. Other media have been found which can support the life of all the pathogenic bacteria isolated. These consist of proteids or carbohydrates in a fluid, semi-solid, or solid form, in a transparent or opaque condition. The advantage of having a variety of media lies in the fact that growth characters on particular media, non-growth on some and growth on others, etc., constitute specific differences which are valuable in the identification of bacteria. The most commonly used media have as their basis a watery extract of meat. Most bacteria in growing in such an extract cause only a grey turbidity. A great advance resulted when Koch, by adding to it gelatin, provided a transparent solid medium in which growth characteristics of particular bacteria

become evident. Many organisms, however, grow best at a temperature at which this nutrient gelatin is fluid, and therefore another gelatinous substance called agar, which does not melt below  $98^{\circ}\text{C}.$ , was substituted. Bouillon made from meat extract, gelatin, and agar media, and the modifications of these, constitute the chief materials in which bacteria are grown.

### *Preparation of Meat Extract.*

The flesh of the ox, calf, or horse is usually employed. Horse-flesh has the advantage of being cheaper and containing less fat than the others; though generally quite suitable, it has the disadvantage for certain purposes of containing a larger proportion of fermentable sugar. The flesh must be freed from fat, and finely minced. To a pound of mince add 1000 c.c. distilled water, and mix thoroughly in a shallow dish. Set aside in a cool place for twenty-four hours. Skim off any fat present, removing the last traces by stroking the surface of the fluid with pieces of filter paper. Place a clean linen cloth over the mouth of a large filter funnel, and strain the fluid through it into a flask. Pour the minced meat into the cloth, and gathering up the edges of the latter in the left hand, squeeze out the juice still held back in the contained meat. Finish this expression by putting the cloth and its contents into a meat press (Fig. 6), similar to that used by pharmacists in preparing extracts; thus squeeze out the last drops. The resulting sanguineous fluid contains the soluble albumins of the meat, the soluble salts, extractives, and colouring matter, chiefly hæmoglobin. It is now boiled thoroughly for two hours, by which process the albumins coagulable by heat are coagulated. Strain now through a clean cloth, boil for another half hour, and filter through white Swedish filter paper (best, C. Schleicher u. Schull, No. 595). Make up to 1000 c.c. with distilled water. The resulting fluid ought to be quite transparent, of a yellowish colour without any red tint. If there is any redness, the fluid must be reboiled and filtered till this colour disappears, otherwise in the later stages it will become opalescent. A large quantity of the extract may be made at a time, and what is not

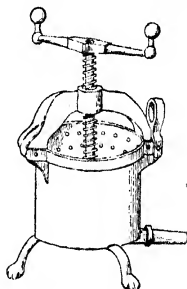


FIG. 6.—Meat press.

## 32 METHODS OF CULTIVATION OF BACTERIA

immediately required is put into a large flask, the neck plugged with cotton wool, and the whole sterilised by methods B (2) or (3). This extract contains very little albuminous matter, and consists chiefly of the soluble salts of the muscle, certain extractives, and altered colouring matters, along with any slight traces of soluble proteid not coagulated by heat. It is of acid reaction. We have now to see how, by the addition of proteid and other matter, it may be transformed into proper culture media.

**1. Bouillon Media.**—These consist of meat extract with the addition of certain substances to render them suitable for the growth of bacteria.

(1) (a). **Peptone Broth or Bouillon.**—This has the composition:—

|                 |   |   |   |   |           |
|-----------------|---|---|---|---|-----------|
| Meat extract    | . | . | . | . | 1000 c.c. |
| Sodium chloride | . | . | . | . | 5 grms.   |
| Peptone albumin | . | . | . | . | 10 „      |

Boil till the ingredients are quite dissolved, and neutralise with a saturated solution of sodium hydrate. Add the latter drop by drop, shaking thoroughly between each drop and testing the reaction by means of litmus paper. Go on till the reaction is slightly but distinctly alkaline. Neutralisation must be practised with great care, as under certain circumstances, depending on the relative proportions of the different phosphates of sodium and potassium, what is known as the amphoteric reaction is obtained, *i.e.* red litmus is turned blue, and blue red, by the same solution. The sodium hydrate must be added till red litmus is turned slightly but distinctly blue, and blue litmus is not at all tinted red. After alkalinisation, allow the fluid to become cold, filter through Swedish filter paper into flasks, make up to original volume with distilled water, plug the flasks with cotton wool, and sterilise by methods B (2) or (3), (pp. 27, 29). This method of neutralisation is to be recommended for all ordinary work.

In this medium the place of the original albumins of the meat is taken by peptone, a soluble proteid not coagulated by heat. Here it may be remarked that the commercial peptone albumin is not pure peptone, but a mixture of albumoses (see footnote, p. 165) with a variable amount of pure peptone. The addition of the sodium chloride is necessitated by the fact that alkalinisation precipitates some of the phosphates and carbonates present. Experience has shown that sodium chloride can quite well be substituted. The reason for the alkalinisation is that it is found that most bacteria grow best on a medium slightly alkaline to litmus. Some, *e.g.* the cholera vibrio, will not grow at all on even a slightly acid medium.

**Standardisation of Reaction of Media.**—While the above procedure of dealing with the reaction of a medium is sufficient for ordinary work, it has been thought advisable to have a more exact method for making media to be used in growing organisms, the growth characteristics of which are to be described for systematic purposes. Such a method should also be used in studying the changes in reaction produced in a medium by the growth of bacteria. It, however, involves considerable difficulty, and should not be undertaken by the beginner. It entails the preparation of solutions of acid and alkali which may be used for determining the original reaction of the medium, and for accurately making it of a definite degree of alkalinity. Normal<sup>1</sup> and decinormal solutions of sodium hydrate and hydrochloric acid are used.

**Preparation of Standard Solutions.**—The first requisites here are normal solutions of acid and alkali. The latter is prepared as follows:—85 grammes of pure sodium bicarbonate are heated to dull redness for ten minutes in a platinum vessel and allowed to cool in an exsiccator; just over 54 grammes of sodium carbonate should now be present. Any excess is quickly removed, and the rest being dissolved in one litre of distilled water, a normal solution is obtained. A measured quantity is placed in a porcelain dish, and a few drops of a .5 per cent solution of phenol-phthaleine in neutral methylated spirit is added to act as indicator. The alkali produces in the latter a brilliant rose pink which, however, disappears on the least excess of acid being present. The mixture is boiled and a solution of hydrochloric acid of unknown strength is run into the dish from a burette till the colour goes and does not return after very thorough stirring. The strength of the acid can then be calculated, and a normal solution can be obtained. From these two solutions any strength of acid or alkali (such as the decinormal solution of NaOH mentioned below) may be derived.

As Eyre has suggested, the reaction of a medium may be conveniently expressed by the sign + or - to indicate acid or alkaline respectively, and a number to indicate the number of cubic centimetres of normal acid or alkaline solution necessary to make a litre of the medium neutral to phenol-phthaleine. Thus, for example, "reaction = - 15," will mean that the medium is alkaline, and requires 15 c.c. of normal HCl to make a litre

<sup>1</sup> A "normal" solution of any salt is prepared by dissolving an "equivalent" weight in grammes of that salt in a litre of distilled water. If the metal of the salt be monovalent, *i.e.* if it be replaceable in a compound by one atom of hydrogen (*e.g.* sodium), an equivalent is the molecular weight in grammes. In the case of NaCl, it would be 58.5 grammes (atomic weight of Na = 23, of Cl = 35.5). If the metal be bivalent, *i.e.* requiring two atoms of H for its replacement in a compound (*e.g.* calcium), an equivalent is the molecular weight in grammes divided by two. Thus in the case of CaCl<sub>2</sub> an equivalent would be 55.5 grammes (atomic weight of Ca = 40, of Cl<sub>2</sub> = 71).



## 34 METHODS OF CULTIVATION OF BACTERIA

neutral. It has been found that when a medium such as bouillon reacts neutral to litmus, its reaction to phenol-phthaleine, according to the above standard, is on the average +25. Now as litmus was originally introduced by Koch, and as nearly all bacterial research has been done with media tested by litmus, it is evidently difficult to say exactly what precise degree of alkalinity is the optimum for bacterial growth. It is probably safe to say, however, that when a medium has been rendered neutral to phenol-phthaleine by the addition of NaOH, the optimum degree is generally attained by the addition of from 10 to 15 c.c. of normal HCl per litre, *i.e.* the optimum reaction is +10 to +15. In other words, the optimum reaction for bacterial growth lies, as Fuller has pointed out, about midway between the neutral point indicated by phenol-phthaleine and the neutral point indicated by litmus.

The only objection to the use of phenol-phthaleine is that its action is somewhat vitiated if free  $\text{CO}_2$  be present. This can be completely obviated as follows. Before testing any medium it is boiled in the porcelain dish into which titration takes place. The soda solutions are best stored in bottles such as that shown in Fig. 44, having on the air inlet a little bottle filled with soda lime with tubes fitted as in the large one. The  $\text{CO}_2$  of the air which passes through is thus removed.

*Method.*—The following procedure includes most of the improvements introduced by Eyre. The medium with all its constituents dissolved is filtered and then heated for about 45 minutes in the steamer, the maximum acidity being reached after this time. Of the warm medium take 25 c.c. and put in a porcelain dish, add 25 c.c. distilled water, and 1 c.c. phenol-phthaleine solution. Run in decinormal soda till neutral point is reached, indicated by the first trace of pink colour, the mixture being kept hot.<sup>1</sup> Repeat process thrice, and take the mean; this divided by 10 will give the amount ( $x$ ) of *normal* soda required to neutralise 25 c.c. of medium; then  $40x$  = amount necessary to neutralise a litre; and  $40x - 10$  = amount of normal soda necessary to give a litre its optimum reaction. Then measure the amount of medium to be dealt with, and add the requisite amount of soda solution.

<sup>1</sup> The beginner may find considerable difficulty in recognising the first tint of pink in the yellow bouillon. A good way of getting over this is to take two samples of the medium, adding the indicator to one only; then to run the soda into these from separate burettes; for each few drops run into the medium containing the indicator the same amount is run into the other. Thus the recognition of the first permanent change in tint will be at once recognised by comparing the two lots of solution.

Eyre uses a soda solution of ten times normal strength, which is delivered out of a 1 c.c. pipette divided into hundredths; this obviates, to a large extent, the error introduced by increasing the bulk of the medium on the addition of the neutralising solution.

1 (b). **Glucose Broth.**—To the other constituents of 1 (a) there is added 1 or 2 per cent of grape sugar. The steps in the preparation are the same. Glucose being a reducing agent, no free oxygen can exist in a medium containing it, and therefore glucose broth is used as a culture fluid for anaerobic organisms.

1 (c). **Glycerin Broth.**—The initial steps are the same as in 1 (a), but *after filtration* 6 to 8 per cent of glycerin (sp. grav. 1.25) is added. This medium is especially used for growing the tubercle bacillus when the soluble products of the growth of the latter are required.

2. **Gelatin Media.**—These are simply the above broths, with gelatin added as a solidifying body.

2 (a). **Peptone Gelatin** :—

|                           |            |
|---------------------------|------------|
| Meat extract . . . . .    | 1000 c.c.  |
| Sodium chloride . . . . . | 5 grms     |
| Peptone albumin . . . . . | 10 "       |
| Gelatin . . . . .         | 100-150 .. |

(the "gold label" gelatin of Coignet et Cie, Paris, is the best). The gelatin is cut into small pieces, and added with the other constituents to the extract; they are then thoroughly melted on a sand bath, or in the "Koch." The fluid medium is then rendered slightly alkaline, as in 1 (a), and filtered through filter paper. As the medium must not be allowed to solidify during the process, it must be kept warm. This is effected by putting the flask and funnel into a tall Koch's steriliser, in which case the funnel must be supported on a tripod or diaphragm, as there is great danger of the neck of the flask breaking if it has to support the funnel and its contents. The filtration may also be carried out in a funnel with water-jacket which is heated, as shown in Fig. 7. Whichever instrument be used, before filtering shake up the melted medium, as it is apt

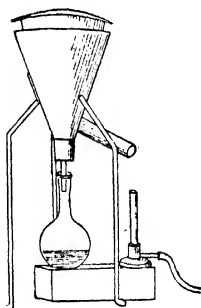


FIG. 7.—Hot-water funnel.

while melting to have settled into layers of different density. Sometimes what first comes through is turbid. If so, replace it in the unfiltered part: often the subsequent filtrate in such circumstances is quite clear. A litre flask of the finished product ought to be quite transparent. If, however, it is partially opaque, add the white of an egg, shake up well, and boil thoroughly over the sand bath. The consequent coagulation of the albumin carries down the opalescent material, and on making up with distilled water to the original quantity and refiltering, it will be found to be clear. The flask containing it is then plugged with cotton wool and sterilised, best by method B (2), p. 27. If the autoclave be used the temperature employed must not be above  $105^{\circ}\text{C}$ ., and exposure not more than a quarter of an hour on three successive days. Too much boiling, or boiling at too high a temperature, as has been said, causes a gelatin medium to lose its property of solidification. The exact percentage of gelatin used in its preparation depends on the temperature at which growth is to take place. Its firmness is its most valuable characteristic, and to maintain this in summer weather, 15 parts per 100 are necessary. A limit is placed on higher percentages by the fact that, if the gelatin be too stiff, it will split on the perforation of its substance by the platinum needle used in inoculating it with a bacterial growth; 15 per cent gelatin melts at about  $24^{\circ}\text{C}$ .

2 (b). **Glucose Gelatin.**—The constituents are the same as 2 (a), with the addition of 1 to 2 per cent of grape sugar. The method of preparation is identical. This medium is used for growing anaerobic organisms at the ordinary temperatures.

3. **Agar Media (French, "gélose").**—The disadvantage of gelatin is that at the blood temperature ( $38^{\circ}\text{C}$ .), at which most pathogenic organisms grow best, it is liquid. To get a medium which will be solid at this temperature, agar is used as the stiffening agent instead of gelatin. Unlike the latter, which is a proteid, agar is a carbohydrate. It is derived from the stems of various sea-weeds growing in the Chinese seas, popularly classed together as "Ceylon Moss." For bacteriological purposes the dried stems of the seaweed may be used, but there is in the market a purified product in the form of a powder; this is preferable.

3 (a). **"Ordinary" Agar.**—This has the following composition:—

|                 |   |   |   |   |   |           |
|-----------------|---|---|---|---|---|-----------|
| Meat extract    | . | . | . | . | . | 1000 c.c. |
| Sodium chloride | . | . | . | . | . | 5 grms.   |
| Peptone albumin | . | . | . | . | . | 10 "      |
| Agar            | . | . | . | . | . | 15 "      |

Cut up the agar into very fine fragments (in fact till it is as nearly as possible dust), add to the meat extract with the other ingredients, and preferably allow to stand all night. Then boil gently in a water bath for two or three hours, till the agar is thoroughly melted. The process of melting may be hastened by boiling the medium in a sand bath and passing through it a stream of steam generated in another flask; the steam is led from the second flask by a bent glass tube passing from just beneath the cork to beneath the surface of the medium (Eyre). After melting render slightly alkaline with sodium hydrate solution, make up to original volume with distilled water, and filter. Filtration here is a very slow process and is best carried out in a tall Koch's steriliser. In doing this, it is well to put a glass plate over the filter funnel to prevent condensation water from dropping off the roof of the steriliser into the medium. If a slight degree of turbidity may be tolerated, it is sufficient to filter through a felt bag or jelly strainer. Plug the flask containing the filtrate, and sterilise either in autoclave for fifteen minutes or in Koch's steriliser for one and a half hours. Agar melts just below  $100^{\circ}\text{C}$ ., and on cooling solidifies about  $39^{\circ}\text{C}$ .

3 (b). **Glycerin Agar.**—To 3 (a) after filtration add 6 to 8 per cent of glycerin and sterilise as above. This is used especially for growing the tubercle bacillus.

3 (c). **Glucose Agar.**—Prepare as in 3 (a), but add 1 to 2 per cent of grape sugar along with agar. This medium is used for the culture of anaerobic organisms at temperatures above the melting-point of gelatin. It is also a superior culture medium for some aerobes, e.g. the *b. diphtheriae*.

These bouillon, gelatin, and agar preparations constitute the most frequently used media. Growths in bouillon do not usually show any characteristic appearances which facilitate classification, but such a medium is of great use in investigating the soluble toxic products of bacteria. The most characteristic developments of organisms take place on the gelatin media. These have, however, the disadvantage of not being available when growth is to take place at any temperature above  $24^{\circ}\text{C}$ . For higher temperatures agar must be employed. Agar is, however, never so transparent. Though quite clear when fluid, on solidifying it always becomes slightly opaque. Further, growths upon it are never so characteristic as those on gelatin. It is, for instance, never liquefied, whereas some organisms, by their growth, liquefy gelatin and others do not—a fact of prime importance.

**Litmus Media.**—To any of the above media litmus (French,

tournesol) may be added to show change in reaction during bacterial growth. The litmus is added, before sterilisation, as a strong watery solution (*e.g.* the Kubel-Tiemann solution, *vide* p. 42) in sufficient quantity to give the medium a distinctly bluish tint. During the development of an acid reaction the colour changes to a pink and may subsequently be discharged.

*Use of neutral red.*—This dye has been introduced as an aid in determining the presence or absence of members of the *b. coli* group, especially in the examination of water. The media found most suitable are agar or bouillon containing .5 per cent of glucose, to which .5 per cent of a one per cent watery solution of neutral red is added. The use of these media and their probable value are described below (*vide* Typhoid Fever).

**Blood Agar : Serum Agar.**—The former medium was introduced by Pfeiffer for growing the influenza bacillus, and it has been used for the organisms which are not easily grown on the ordinary media, *e.g.* the gonococcus and the pneumococcus. Human blood or the blood of animals may be used. "Sloped tubes" (*vide* p. 48) of agar are employed (glycerin agar is not so suitable). Purify a finger first with 1-1000 corrosive sublimate, dry, and then wash with absolute alcohol to remove the sublimate. Allow the alcohol to evaporate. Prick with a needle sterilised by heat, and, catching a drop of blood in the loop of a sterile platinum wire (*vide* p. 49), smear it on the surface of the agar. The excess of the blood runs down and leaves a film on the surface. Cover the tubes with india-rubber caps, and incubate them for one or two days at 38° C. before use, to make certain that they are sterile. Agar poured out in a thin layer in a Petri dish may be smeared with blood in the same way and used for cultures. A medium composed of one part of fresh blood (drawn aseptically) and two parts of fluid agar at 40° C., has been used for the cultivation of the bacillus of soft sore.

*Serum agar* is prepared in a similar way by smearing the surface of the agar with blood serum, or by adding a few drops of serum to the tube and then allowing it to flow over the surface.

#### *Peptone Solution.*

A simple solution of peptone (Witte) constitutes a suitable culture medium for many bacteria. The peptone in the proportion of 1 to 2 per cent, along with .5 per cent NaCl, is dissolved in distilled water by heating. The fluid is then filtered, placed in tubes and sterilised. The reaction is usually distinctly alkaline, which condition is suitable for most purposes. For

special purposes the reaction may be standardised. In such a solution the cholera vibrio grows with remarkable rapidity. It is also much used for testing the formation of indol by a particular bacterium; and by the addition of one of the sugars to it the fermentative powers of an organism may be tested (p. 75). Litmus may be added to show any change in reaction.

### *Blood Serum.*

Koch introduced this medium, and it is prepared as follows: Plug the mouth of a tall cylindrical glass vessel (say of 1000 c.c. capacity) with cotton wool, and sterilise by steaming it in a Koch's steriliser for one and a half hours. Take it to the place where a horse, ox, or sheep is to be killed. When the artery or vein of the animal is opened, allow the first blood which flows, and which may be contaminated from the hair, etc., to escape; fill the vessel with the blood subsequently shed. Carry carefully back to the laboratory without shaking, and place for twenty-four hours in a cool place, preferably an ice chest. The clear serum will separate from the clotted blood. If a centrifuge is available, a large yield of serum may be obtained by centrifugalising the freshly drawn blood. If coagulation has occurred, the clot must first be thoroughly broken up. With a sterile 10 c.c. pipette, transfer this quantity of serum to each of a series of test-tubes which must previously have been sterilised by dry heat. The serum may, with all precautions, have been contaminated during the manipulations, and must be sterilised. As it will coagulate if heated above 68° C., advantage must be taken of the intermittent process of sterilisation at 57° C. [method B (4)]. It is therefore kept for one hour at this temperature on each of eight successive days. It is always well to incubate it for a day at 37° C. before use, to see that the result is successful. After sterilisation it is "inspissated," by which process a clear solid medium is obtained. "Inspissation" is probably an initial stage of coagulation, and is effected by keeping the serum at 65° C. till it stiffens. This temperature is just below the coagulation point of the serum. The more slowly the operation is performed the clearer will be the serum. The apparatus used for the purpose is one of the various forms of serum steriliser (*e.g.* Fig. 8), generally a chamber with water-jacket heated with a Bunsen below. The temperature is controlled by a gas regulator, and such an apparatus can, by altering the temperature, be used either for sterilisation or inspissation. As is evident, the preparation of this medium is tedious, but its

## 40 METHODS OF CULTIVATION OF BACTERIA

use is necessary for the observation of particular characteristics in several pathogenic bacteria, notably the tubercle bacillus. Pleuritic and other effusions may be prepared in the same way, and used as media, but care must be taken in their use, as we have no right to say that pathological effusions have the same chemical composition as normal serum.

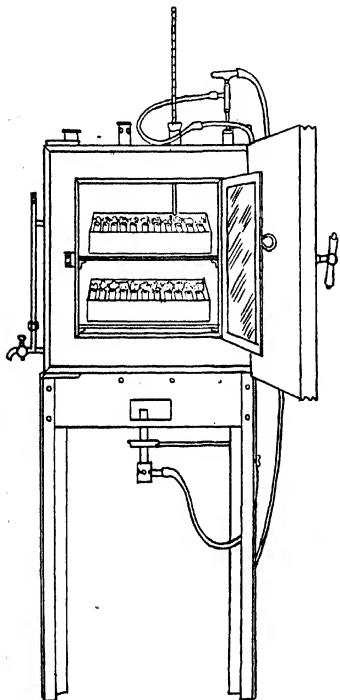


FIG. 8.—Blood serum inspissator.

If blood be collected with strict aseptic precautions, then sterilisation of the serum is unnecessary. To this end the mouth of the cylinder used for collecting the blood, instead of being plugged with wool, has an india-rubber bung inserted in it through which two bent glass tubes pass. The outer end of one of these is of convenient length, and, before sterilisation, a large cap of cotton wool is tied over it; the other tube is plugged with a piece of cotton wool. In the slaughter-house the cap is removed and the tube is inserted into the blood-vessel as a cannula. The cylinder is thus easily filled. Another method is to conduct the blood to the cylinder by means of a sterilised cannula and india-rubber tube, the former being inserted in

the blood-vessel. In every case the serum must be incubated before use, to make sure that it is sterile.

**Coagulated Blood Serum.**—If fresh serum be placed in sterile tubes and be steamed in the sloped position for an hour it coagulates, and there is thus obtained a solid medium very useful for the growth of the diphtheria bacillus for diagnostic purposes.

**Löffler's Blood Serum.**—This is the best medium for the

growth of the *b. diphtheriæ* and may be used for other organisms. It has the following composition. Three parts of calf's or lamb's blood serum are mixed with one part ordinary neutral peptone bouillon made from veal with 1 per cent of grape sugar added to it. Though this is the original formula it can be made from ox or sheep serum and beef bouillon without its qualities being markedly impaired. Sterilise by method B (4) as above (p. 29).

**Alkaline Blood Serum** (Lorrain Smith's Method).—To each 100 c.c. of the serum obtained as before, add 1 to 1·5 c.c. of a 10 per cent solution of sodium hydrate and shake gently. Put sufficient of the mixture into each of a series of test-tubes, and laying them on their sides, sterilise by method B (2). If the process of sterilisation be carried out too quickly, bubbles of gas are apt to form before the serum is solid, and these interfere with the usefulness of the medium. Dr. Lorrain Smith informs us that this can be obviated if the serum be solidified high up in the Koch's steriliser, in which the water is allowed only to simmer. In this case sterilisation ought to go on for one and a half hours. A clear solid medium (consisting practically of alkali-albumin) is thus obtained, and he has found it of value for the growth of the organisms for which Koch's serum is used, and especially for the growth of the *b. diphtheriæ*. Its great advantage is that aseptic precautions in obtaining blood from the animal are not necessary, and it is easily sterilised.

**Marmorek's Serum Media.**—There has always been a difficulty in maintaining the virulence of cultures of the pyogenic streptococci, but Marmorek has succeeded in doing so by growing them on the following media, which are arranged in the order of their utility :—

1. Human serum 2 parts, bouillon 1 part.
2. Pleuritic or ascitic serum 1 part, bouillon 2 parts.
3. Asses' or mules' serum 2 parts, bouillon 1 part.
4. Horse serum 2 parts, bouillon 1 part.

Human serum can be obtained from the blood shed in venesection, the usual aseptic precautions being taken. In the case of these media, sterilisation is effected by method B (4), and they are used fluid.

**Hiss's Serum Water Media.**—These are composed of one part of ox's serum and three parts of distilled water with 1 per cent litmus; various sugars in a pure condition are added in the proportion of 1 per cent. The development of acid by fermentation is shown by the alteration of the colour and by



## 42 METHODS OF CULTIVATION OF BACTERIA

coagulation of the medium. These media do not coagulate at 100° C. and thus can be sterilised in the steam steriliser. They have been extensively used by American workers in studying the fermentative properties of the *b. dysenteriae*, *b. coli*, etc.

**Drigalski and Conradi's Medium.**—This is one of the media used for the study of intestinal bacteria and especially for the isolation of the typhoid group of organisms. (a) Three pounds of meat are treated with two litres of water overnight; the fluid is separated as usual, boiled for an hour, filtered, and there are added 20 grammes Witte's peptone, 20 grammes nutrose,<sup>1</sup> 10 grammes sodium chloride; the mixture is then boiled for an hour, 60 grammes finest agar are added, and it is placed in the autoclave till melted (usually one hour); it is then rendered slightly alkaline to litmus, filtered, and boiled for half an hour. (b) 260 c.c. Kubel-Tiemann litmus<sup>2</sup> solution is boiled for ten minutes, 30 grammes milk sugar (chemically pure) are added, and the mixture is boiled for fifteen minutes; (a) and (b) are then mixed hot, well shaken, and, if necessary, the slightly alkaline reaction restored. There are then added 4 c.c. of a 10 per cent sterile solution of water-free sodium hydrate and 20 c.c. of a freshly prepared solution made by dissolving .1 gramme crystal-violet B, Hoechst, in 100 c.c. hot sterile distilled water. This is the finished medium, and great care must be taken not to overheat it or to heat it too long, as changes in the lactose may be originated. It is convenient to distribute the medium in 80 c.c. flasks.

The principle of the medium is that while there is a food supply very favourable to the *b. typhosus* and the *b. coli* the antiseptic action of the crystal-violet tends to inhibit the growth of other bacteria likely to occur in material which has been subjected to intestinal contamination. In examining faeces a little is rubbed up in from ten to twenty times its volume of sterile normal salt solution; in the case of urine or water the fluid is centrifugalised and the deposit or lower portion is used for the inoculation procedures.

For use the medium is distributed in Petri capsules in a rather thicker layer than is customary in an ordinary plate. The sheet of medium must be transparent, but must not be less than 2 mm. in thickness—in fact, ought to be about 4 mm. After being poured, the capsules are left with the covers off for an hour or so, to allow the superficial layers of the medium to become set hard. The effect of this is that during incubation no water of condensation forms on the lid of the capsule, and thus the danger of this fluid dropping on to the developing colonies is avoided. The antiseptic nature of the crystal-violet is sufficient to prevent the growth of any aerial organisms falling

<sup>1</sup> Nutrose is an alkaline preparation of casein.

<sup>2</sup> The litmus solution is made as follows. Solid commercial litmus is digested with pure spirit at 30° C. till on adding fresh alcohol the latter becomes only of a light violet colour. A saturated solution of the residue is then made in distilled water and filtered. When this is diluted with a little distilled water it is of a violet colour, which further dilution turns to a pure blue. To such a blue solution very weak sulphuric acid (made by adding two drops of dilute sulphuric acid to 200 c.c. water) is added till the blue colour is turned to a wine-red. Then the saturated solution of the dye is added till the blue colour returns.

on the agar during its exposure to the air. The plates are usually inoculated by means of a glass spatula made by bending three inches of a piece of glass rod at right angles to the rest of the rod. This part is dipped in the infective material and smeared in all directions over the surfaces of three or four plates successively without any intervening sterilisation. The plates are again exposed to the air after inoculation for half an hour and then incubated for twenty-four hours. At the end of such a period *b. coli* colonies are 2 to 6 mm. in diameter, stained distinctly red, and are non-transparent. Colonies of the *b. typhosus* are seldom larger than 2 mm., they are blue or bluish-violet in colour, are glassy and dew-like in character, and have a single contour. Sometimes in the plates *b. subtilis* and its congeners appear, and colonies of these organisms have a blue colour. Their growth is, however, more exuberant than that of the typhoid bacillus,—being often heaped up in the centre, —and the contour of the colony is often double.

**MacConkey's Bile-Salt Media.**—These media were introduced for the purpose of differentiating the intestinal bacteria, and have been extensively used for the study of the *b. coli*, *b. typhosus*, *b. dysenteriae*, etc. The characteristic ingredients are bile salts and various sugars. The stock solution is the following:—Commercial sodium taurocholate, 0·5 gramme; Witte's peptone, 2·0 grammes; distilled water, 100 c.c. For a liquid medium there is added to this '5 per cent of a freshly prepared 1 per cent solution of neutral red<sup>1</sup> and the sugar,—when glucose is used 0·5 per cent is added, in the case of other sugars 1 per cent. The fluid is distributed in Durham's fermentation tubes and sterilised in the steamer for ten minutes on two successive days, care being taken not to overheat the medium.

For bile-salt agar 1·5 to 2 per cent agar is dissolved in the stock solution in the autoclave, if necessary cleared with white of egg and filtered. Neutral red and a sugar are added, as in the case of the liquid medium. As with Drigalski's medium, it is well to sterilise it in flasks containing 80 c.c., this being an amount sufficient for three Petri capsules. When this medium is used for examining urine or fæces, plates are inoculated as with Drigalski's medium (*supra*); for its use in water examinations see p. 136.

In the bile-salt bouillon the formation of both acid and gas is observed if such formation occurs, and in the bile-salt agar acid production is recognised by the red colour of the colonies of the acid-producing organisms.

MacConkey's original medium was a 1 per cent bile-salt lactose agar with no indicator, and was used for the detection of intestinal bacteria in water. Such a medium is unfavourable to all the common spore-bearing organisms found in water, and by incubating at 42° C. tubes, in which there is probably a mixed infection from such a source, the growth of most other water bacteria is inhibited. *B. coli* and *b. typhosus*, on the other hand, grow readily. With the former the surface colonies are broad, irregular, and flat, of opaque colour, and with a small spot of yellow or orange in the centre, and the colony is surrounded by a haze; the deep colonies are lens-shaped, of orange colour, and are likewise surrounded by a haze. With the typhoid organism at the end of forty-eight hours the surface colonies are small, round, raised, and semi-transparent, while the deep colonies are lens-shaped, white, and have no surrounding

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<sup>1</sup> Neutral red gives a deep crimson with acids and a yellow-red with alkalis.

## 44 METHODS OF CULTIVATION OF BACTERIA.

haze. The haze in the case of *b. coli* is due to the ready production of acid from the lactose causing a precipitate of the taurocholate. Any other organism capable of producing acid from lactose will give a similar reaction, and the haze can be readily cleared up by floating a drop of ammonia on the surface of the medium. MacConkey also used with a similar object a 5 per cent glucose bile-salt bouillon tinted with neutral litmus as in Drigalski's medium.

With reference to MacConkey's fluid media, organisms are divided into (1) those which produce both acid and gas; (2) those producing acid only; (3) those growing but not producing either acid or gas; (4) those incapable of growing. *B. coli* belongs to the first group and *b. typhosus* to the second, and to these groups also belong most ordinary organisms growing in faeces, practically none of which are found in the third and fourth classes. Thus if any growth takes place on this medium when inoculated with, say, water, the probability is that the bacteria have been derived from faeces, but of course their identification might present some difficulty. With the neutral-red solid media the colonies of any organism giving rise to acid will be of a beautiful rose red colour.

**Petruschky's Litmus Whey.**—The preparation of this medium, which is somewhat difficult, is as follows:—Fresh milk is slightly warmed, and sufficient very dilute hydrochloric acid is added to cause precipitation of the casein, which is now filtered off. Dilute sodium hydrate solution is added up to, but not beyond, the point of neutralisation, and the fluid steamed for one to two hours, by which procedure any casein which has been converted into acid albumin by the hydrochloric acid is precipitated. This is filtered off, and a clear, colourless, perfectly neutral fluid should result. Its chief constituent, of course, will be lactose. To this sufficient Kubel-Tiemann solution of litmus is added, the medium is put into tubes and then sterilised. After growth has taken place, the amount of acid formed can be estimated by dropping in standardised soda solution till the tint of an uninoculated tube is reached.

**Media for growing Trichophyta, Moulds, etc.**—1. *Beer Wort Agar.* Take beer wort as obtainable from the brewery and dilute it till it has an s.g. of 1100. Add 1·5 per cent of powdered agar and heat in the Koch till it is dissolved (usually about two hours are necessary). Filter rapidly and fill into tubes. Sterilise in the Koch for twenty minutes on three successive days. If the medium is heated too long it loses the capacity of solidifying.

2. *Sabouraud's medium* (modified). Take 40 grammes maltose and 10 grammes Witte's peptone and dissolve these in one litre of water, then add 13 grammes of powdered agar. Heat in the Koch till the agar is dissolved, filter and fill into tubes, sterilise in the autoclave for twenty minutes at 120° C.

To use these for isolating, say, the *Tinea tonsurans*, pick out an infected hair, wash in absolute alcohol for a few seconds, then wash in changes of sterile water and stab the hair into the surface of the medium in a number of places; incubate at 24° C. Usually it is sufficient to stab the hair as it is picked from the skin into the medium.

### *Potatoes as Culture Material.*

(a) **In Potato Jars.**—The jar consists of a round, shallow, glass vessel with a similar cover (*vide* Fig. 9). It is washed

with 1-1000 corrosive sublimate, and a piece of circular filter paper, moistened with the same, is laid in its bottom. On this latter are placed four sterile watch glasses. Two firm, healthy, small, round potatoes, as free from eyes as possible, and with the skin whole, are scrubbed well with a brush under the tap and steeped for two or three hours in 1-1000 corrosive sublimate. They are steamed in the Koch's steriliser for thirty minutes or longer, or in the autoclave for a quarter of an hour. When cold, each is grasped

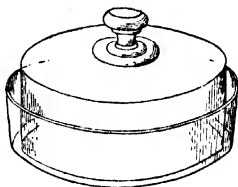


FIG. 9.—Potato jar.



FIG. 10.—Cylinder of potato cut obliquely.

between the left thumb and forefinger (which have been sterilised with sublimate) and cut through the middle with a sterile knife. It is best to have the cover of the jar raised by an assistant, and to perform the cutting beneath it. Each half is put in one of the watch glasses, the cut surfaces, which are then ready for inoculation with a bacterial growth, being uppermost. Smaller jars, each of which holds half of a potato, are also used in the same way and are very convenient.

(b) **By Slices in Tubes.**—This method, introduced by Ehrlich, is the best means of utilising potatoes as a medium. A large, long potato is well washed and scrubbed, and peeled with a clean knife. A cylinder is then bored from its interior with an apple corer or a large cork borer, and is cut obliquely, as in Fig. 10. Two wedges are thus obtained, each of which is placed broad end downward in a test-tube of special form (see Fig. 11). In the wide part at the bottom of this tube is placed a piece of cotton wool, which catches any condensation water which may form. The wedge rests on the constriction above this bulbous portion. The tubes, washed, dried, and with cotton wool in the bottom and in the mouth, are sterilised before the slices of potato are introduced. After the latter are inserted, the tubes are sterilised in the Koch steam steriliser for one hour, or in the autoclave for fifteen minutes, at 115° C. An ordinary test-



FIG. 11.  
—Ehrlich's tube containing piece of potato.

## 46 METHODS OF CULTIVATION OF BACTERIA

tube may be used with a piece of sterile absorbent wool in its bottom, on which the potato may rest.

*Glycerin potato*, suitable for the growth of the tubercle bacillus, may be prepared by covering the slices in the tubes with 6 per cent solution of glycerin in water and steaming for half an hour. The fluid is then poured off and the sterilisation continued for another half hour.

Potatoes ought not to be prepared long before being used, as the surface is apt to become dry and discoloured. It is well to take the reaction of the potato with litmus before sterilisation, as this varies; normally in young potatoes it is weakly acid. The reaction of the potato may be more accurately estimated by steaming the potato slices for a quarter of an hour in a known quantity of distilled water and then estimating the reaction of the water by phenol-phthaleine. The required degree of acidity or alkalinity is obtained by adding the necessary quantity of HCl or NaOH solution (p. 34) and steaming for other fifteen minutes. The water is then poured off and sterilisation continued for another half hour. Potatoes before being inoculated ought always to be incubated at 37° C. for a night, to make sure that their sterilisation has been successful.

**Elsner's Medium.**—This is one of the media introduced in the study of the comparative reactions of the typhoid bacillus and the *B. coli*. The preparation is as follows: 500 grammes potato are grated up in a litre of water, allowed to stand over night, then strained, and added to an equal quantity of ordinary 15 per cent peptone gelatin which has not been neutralised. Normal sodium hydrate solution is added till the reaction is feebly acid to litmus, the whole boiled together, filtered, and sterilised. Just before use potassium iodide is added so as to constitute one per cent of the medium. Moore has used a similar agar preparation. Here 500 grammes potato are scraped up in one litre of water, allowed to stand for three hours, strained, and put aside over night. The clear fluid is poured off, made up to one litre, rendered slightly alkaline, 20 grammes agar are added, and the whole is treated as in making ordinary agar. The medium is distributed in test-tubes—10 c.c. to each—and immediately before use, to each is added 5 c.c. of a solution of 10 grammes potassium iodide to 50 c.c. water.

### *Milk as a Culture Medium.*

This is a convenient medium for observing the effects of bacterial growth in changing the reaction, in coagulating the soluble albumin, and in fermenting the lactose. It is prepared as follows: fresh milk is taken, preferably after having had the cream "separated" by centrifugalisation, as is practised in the best dairies, and is steamed for fifteen minutes in the Koch, it is then set aside in an ice chest or cool place over night to

facilitate further separation of cream. The milk is siphoned off from beneath the cream. The reaction of fresh milk is alkaline. If great accuracy is necessary any required degree of reaction may be obtained by the titration method. It is then placed in tubes and sterilised by methods B (2) or B (3).

### *Bread Paste.*

This is useful for growing torulæ, moulds, etc. Some ordinary bread is cut into slices, and then dried in an oven till it is so dry that it can be pounded to a fine powder in a mortar, or rubbed down with the fingers and passed through a sieve. Some 100 c.c. flasks are washed, dried, and sterilised, and a layer of the powder half an inch thick placed on the bottom. Distilled water, sufficient to cover the whole of it, is then run in with a pipette held close to the surface of the bread, and, the cotton-wool plugs being replaced, the flasks are sterilised in the Koch's steriliser by method B (2). The reaction is slightly acid.

## THE USE OF THE CULTURE MEDIA.

The culture of bacteria is usually carried on in test-tubes conveniently  $6 \times \frac{5}{8}$  in. These ought to be very thoroughly washed and dripped, and their mouths plugged with plain cotton wool. They are then sterilised for one hour at  $170^{\circ}$  C. If the tubes be new, the glass, being usually packed in straw, may be contaminated with the extremely resisting spores of the *b. subtilis*. Cotton-wool plugs are universally used for protecting the sterile contents of flasks and tubes from contamination with the bacteria of the air. A medium thus protected will remain sterile for years. Whenever a protecting plug is removed for even a short time, the sterility of the contents may be endangered. It is well to place the bouillon, gelatin, and agar media in the test-tubes directly after filtration. The media can then be sterilised in the test-tubes.

In filling tubes, care must be taken to run the liquid down the centre, so that none of it drops on the inside of the upper part of the tube with which the cotton-wool plug will be in contact, otherwise the latter will subsequently stick to the glass and its removal will be difficult. In the case of liquid media, test-tubes are filled about one-third full. With the solid media the amount varies. In the case of gelatin media, tubes filled one-third full and allowed to solidify while standing upright, are those commonly used. With

## 48 METHODS OF CULTIVATION OF BACTERIA

organisms needing an abundant supply of oxygen the best growth takes place on the surface of the medium, and for practical purposes the surface ought thus to be as large as possible. To this end "sloped" agar and gelatin tubes are used. To prepare these, tubes are filled only about one-sixth full, and after sterilisation are allowed to solidify, lying on their sides with their necks supported so that the contents extend 3

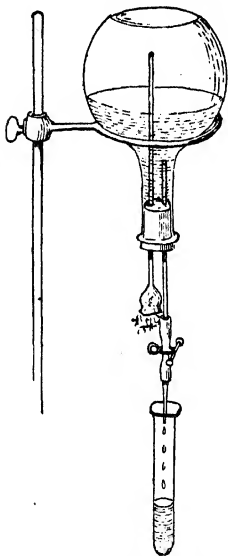


FIG. 12.—Apparatus which may be used for filling tubes. The apparatus explains itself. The india-rubber stopper with its tubes ought to be sterilised before use.

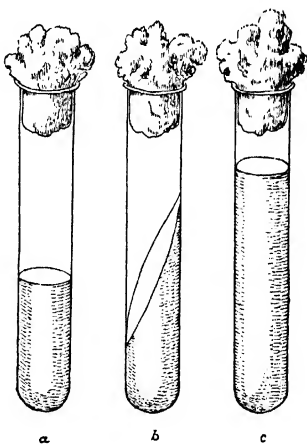


FIG. 13.—Tubes of media.

a. Ordinary upright tube. b. Sloped tube.  
c. "Deep" tube for cultures of anaerobes.

to 4 inches up, giving an oblique surface after solidification. Thus agar is commonly used in such tubes (less frequently gelatin is also "sloped"), and this is the position in which blood serum is inspissated. Tubes, especially those of the less commonly used media, should be placed in large jars provided with stoppers, otherwise the contents are apt to evaporate. A tube of medium which has been inoculated with a bacterium, and on which growth has taken place, is called a "culture." A "pure culture" is one in which only one organism is present. The methods of

obtaining pure cultures will presently be described. When a fresh tube of medium is inoculated from an already existing culture, the resulting growth is said to be a "sub-culture" of the first. All manipulations involving the transference of small portions of growth either from one medium to another, as in the inoculation of tubes, or, as will be seen later, to cover-glasses for microscopic examination, are effected by pieces of platinum wire (Nos. 24 or 27 Birmingham wire gauge—the former being the thicker) fixed in glass rods 8 inches long.<sup>1</sup> Every worker should have three such wires. Two are  $2\frac{1}{2}$  inches long, one of these being straight (Fig. 14, *a*), and the other having a loop turned upon it (Fig. 14, *b*). The latter is referred to as the platinum "loop" or platinum "eyelet," and is used for many purposes.

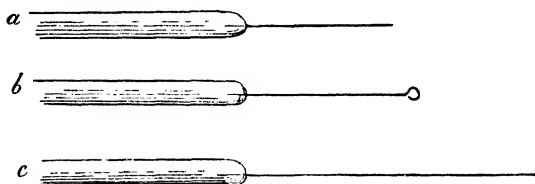


FIG. 14.—Platinum wires in glass handles.

- a.* Straight needle for ordinary puncture inoculations. *b.* "Platinum loop."  
*c.* Long needle for inoculating "deep" tubes.

"Taking a loopful" is a phrase constantly used. The third wire (Fig. 14, *c*) ought to be  $4\frac{1}{2}$  inches long and straight. It is used for making anaerobic cultures. It is also very useful to have at hand a platinum-iridium spud. This consists of a piece of platinum-iridium about  $1\frac{1}{2}$  inches long, 2 mm. broad, and of sufficient thickness to give it a firm consistence; its distal end is expanded into a diamond shape, and its proximal is screwed into an aluminium rod. It is very useful for making scrapings from organs and for disintegrating felted bacterial cultures; in such manipulations the ordinary platinum wire is awkward to work with as it bends so easily. Cultures on a solid medium are referred to (1) as "puncture" or "stab" cultures (German, *Stichkultur*), or (2) as "stroke" or "slant" cultures (*Strichkultur*), according as they are made (1) on tubes solidified in the upright position, or (2) on sloped tubes.

<sup>1</sup> Aluminium rods are made which are very convenient. The end is split with a knife, the platinum wire is inserted and fixed by pinching the aluminium on it in a vice.



## 50 METHODS OF CULTIVATION OF BACTERIA

To inoculate, say, one ordinary upright gelatin tube from another, the two tubes are held in an inverted position between the forefinger and thumb of the left hand with their mouths towards the person holding them; the plugs are twisted round

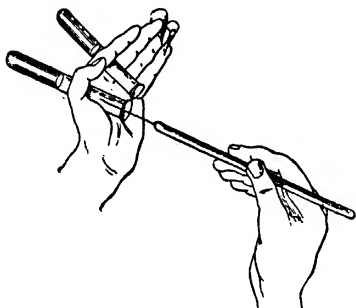


FIG. 15.—Another method of inoculating solid tubes.

once or twice, to make sure they are not adhering to the glass. The short, straight platinum wire is then heated to redness from point to insertion, and 2 to 3 inches of the glass rod are also passed two or three times through the Bunsen flame. It is held between the right fore and middle fingers, with the needle projecting backwards, *i.e.* away from the right palm.

Remove plug from cul-

ture tube with right forefinger and thumb, and continue to hold it between the same fingers, by the part which projected beyond the mouth of the tube. Now touch the culture with the platinum needle, and, withdrawing it, replace plug. In the same way remove plug from tube to be inoculated, and plunge platinum wire down the centre of the gelatin to within half an inch of the bottom. It must on no account touch the glass above the medium. The wire is then immediately sterilised. A variation in detail of this method is to hold the plug of the tube next the thumb between the fore and middle fingers, and the plug of the other between the middle and ring fingers, then to make the inoculation (Fig. 15). If a tube contain a liquid medium, it must be held in a sloping position between the same fingers, as above. For a stroke culture the platinum loop is used, and a little of the culture is smeared in a line along the surface of the medium from below upwards. In inoculating tubes, it is always well, on removing the plugs, to

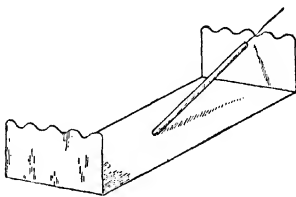


FIG. 16.—Rack for platinum needles.

Remove plug from cul-  
ture tube with right forefinger and thumb, and continue to hold it between the same fingers, by the part which projected beyond the mouth of the tube. Now touch the culture with the platinum needle, and, withdrawing it, replace plug. In the same way remove plug from tube to be inoculated, and plunge platinum wire down the centre of the gelatin to within half an inch of the bottom. It must on no account touch the glass above the medium. The wire is then immediately sterilised. A variation in detail of this method is to hold the plug of the tube next the thumb between the fore and middle fingers, and the plug of the other between the middle and ring fingers, then to make the inoculation (Fig. 15). If a tube contain a liquid medium, it must be held in a sloping position between the same fingers, as above. For a stroke culture the platinum loop is used, and a little of the culture is smeared in a line along the surface of the medium from below upwards. In inoculating tubes, it is always well, on removing the plugs, to

make sure that no strands of cotton fibre are adhering to the inside of the necks. As these might be touched with the charged needle and the plug thus be contaminated, they must be removed by heating the inoculating needle red-hot and scorching them off with it. When the platinum wires are not in use they may be laid in a rack made by bending up the ends of a piece of tin, as in Fig. 16. To prevent contamination of cultures by bacteria falling on the plugs while these are exposed to the air during inoculation manipulations, some bacteriologists singe the plugs in the flame before replacing. This is, however, in most cases a needless precaution. If the top of a plug be dusty it is best to singe it before extraction.

#### THE METHODS OF THE SEPARATION OF AEROBIC ORGANISMS. PLATE CULTURES.

The general principle underlying the methods of separation is the distribution of the bacteria in one of the solid media liquefied by heat and the dilution of the mixture so that the growths produced by the individual bacteria—called colonies—shall be suitably apart. In order to render the colonies easily accessible, the medium is made to solidify in as thin a layer as possible, by being poured out on glass plates—hence the term “plate cultures.”

As the optimum temperature varies with different bacteria, it is necessary to use both gelatin and agar media. Many pathogenic organisms, *e.g.* pneumococcus, *b. diphtheriæ*, etc., grow too slowly on gelatin to allow its ready use. On the other hand, many organisms, *e.g.* some occurring in water, do not develop on agar incubated at 37° C.

**Separation by Gelatin Media.**—As the naked-eye and microscopic appearances of colonies are often very characteristic, plate cultures, besides use in separation, are often taken advantage of in the description of individual organisms. The plate-culture method can also be used to test whether a tube culture is or is not pure. The suspected culture is plated (three plates being prepared, as will be described). If all the colonies are the same, then the cultures may be held to be pure.

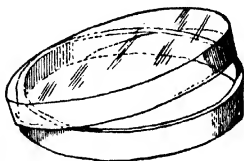


FIG. 17.—Petri's capsule.  
(Cover shown partially raised.)

Either simple plates of glass 4 inches by 3 inches are used,

## 52 METHODS OF CULTIVATION OF BACTERIA

or, what are more convenient, circular glass cells with similar overlapping covers. The latter are known as Petri's dishes or capsules (Fig. 17). They are usually 3 inches in diameter and half an inch deep. The advantage of these is that they do not require to be kept level by a special apparatus while the medium is solidifying, and can be readily handled afterwards without admitting impurities. Whether plates or capsules are used, they are washed, dried with a clean cloth, and sterilised for one hour in dry air at  $170^{\circ}$  C., the plates being packed in sheet-iron boxes made for the purpose see (Fig. 18).

1. *Glass Capsules*.—While in certain circumstances, as when the number of colonies has to be counted, it is best to use plates of glass; in the usual laboratory routine Petri's capsules are to be preferred for the above reasons.

The contents of three gelatin tubes, marked *a*, *b*, *c*,<sup>1</sup> are liquefied by placing in a beaker of water at any temperature between  $25^{\circ}$  C. and  $38^{\circ}$  C. Inoculate *a* with the bacterial mixture. The amount of the latter to be taken varies, and can only be regulated by experience. If the microscope shows enormous numbers of different kinds of bacteria present, just as much as adheres to the point of a straight platinum needle is sufficient. If the number of bacilli is small, one to three loops of the mixture may be transferred to the medium. Shake *a* well, but not so as to cause many fine air-bubbles to form. Transfer two loops of gelatin from *a* to *b*. Shake *b* and transfer five loops to *c*. The plugs of the tubes are in each case replaced and the tubes returned to the beaker. The contents of the three tubes are then poured out into three capsules. In doing so the plug of each tube is removed and the mouth of the tube passed two or three times through the Bunsen flame, the tube being meantime rotated round a longitudinal axis. Any organisms on its rim are thus killed. The capsules are labelled and set aside till growth takes place.

For accurate work it will be found convenient to carry out the dilutions in definite proportions. The following is the procedure which we have found very serviceable. In a number of small sterile test-tubes .95 c.c. sterile water is put. To the first tube we add .05 c.c. of the bacterial mixture. The contents of the tube are well shaken up, and the pipette is sterilised by being washed out with boiling water. It is allowed to cool, and .05 c.c. of fluid is transferred from the first tube to the second. By a similar procedure .05 c.c. is transferred from the second to

<sup>1</sup> For marking glass vessels it is convenient to use the red, blue, or yellow oil pencils made for the purpose by Faber.

the third and so on. There is thus effected a twenty-fold dilution in each successive tube. After these steps have been carried out a definite amount, say, .05 c.c. is transferred from each tube to a tube of melted gelatine,—the gelatine being afterwards plated and the colonies counted when growth occurs. The number of tubes required will vary according to the number of bacteria in the original mixture, but usually four or five will be sufficient. It is quite evident that this method not only enables us to separate bacteria, but if necessary gives us a means of estimating exactly the number in the original mixture.

The colonies appear as minute rounded points, whitish or variously coloured. Their characters can be more minutely studied by means of a hand-lens or by inverting the capsule on the stage of a microscope and examining with a low power through the bottom. From their characters, colour, shape, contour, appearance of surface, liquefaction or non-liquefaction of the gelatin, etc., the colonies can be classified into groups. Further aid in the grouping of the varieties is obtained by making film preparations and examining them microscopically. Gelatin or agar tubes may then be inoculated from a colony of each variety, and the growths obtained are then examined both as to their purity and as to their special characters, with a view to their identification (p. 115).

2. *Glass Plates* (Koch).—When plates of glass are to be used, an apparatus on which they may be kept level while the medium is solidifying is, as has been said, necessary. An apparatus devised by Koch is used (Figs. 18, 19). This consists of a circular plate of glass (with the upper surface ground, the lower polished) on which the plate used for pouring out the medium is placed. The latter is protected from the air during solidification by a bell jar. The circular plate and bell jar rest on the flat rim of a circular glass trough, which is filled quite full with a mixture of ice and water, to facilitate the lowering of the temperature of whatever is placed beneath the bell jar. The glass trough rests on corks on the bottom of a large circular trough, which catches any water that may be spilled. This trough in turn rests on a wooden triangle with a foot at each corner, the height of which can be adjusted, and which thus constitutes the levelling apparatus. A spirit level is placed where the plate is to go, and the level of the ground glass plate thus assured. There is also prepared a "damp chamber," in which the plates are to be stored after being made. This consists of a circular glass trough with a similar cover. It is sterilised by being washed outside and inside with perchloride of mercury 1-1000, and a circle of filter-paper moistened with the same is laid on its bottom. Glass benches on which the plates may be laid are similarly purified.

To separate organisms by this method three tubes, *a*, *b*, *c*, are inoculated as in using Petri's capsules (p. 52). The hands having been washed in perchloride of mercury 1-1000 and dried, the plate box is opened, and a plate lifted by its opposite edges and transferred to the

## 54 METHODS OF CULTIVATION OF BACTERIA

levelled ground glass (as in Figs. 18, 19). The bell jar of the leveller being now lifted a little, the gelatin in tube *a* is poured out on the surface of the sterile plate, and while still fluid, is spread by stroking

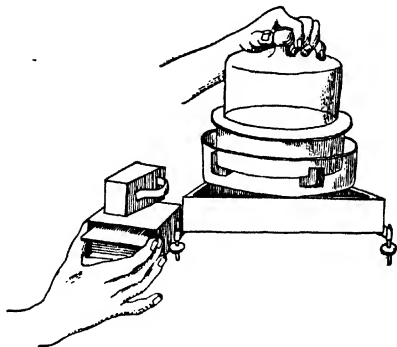


FIG. 18.—Koch's levelling apparatus for use in preparing plates. Hands shown in first position for transferring sterile plate from iron box to beneath bell jar, where it subsequently has the medium poured out upon it.

with the rim of the tube. After the medium solidifies, the plate is transferred to the moist chamber as rapidly as possible, so as to avoid

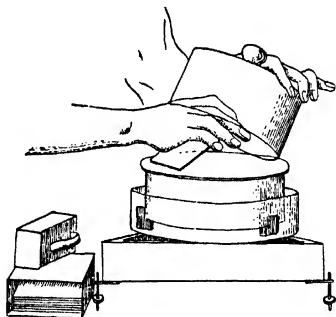


FIG. 19.—Koch's levelling apparatus. Hands shown in second position just as the plate is lowered on to the ground glass surface. By executing the transference of the plate from the box in this way, the surface which was undermost in the latter is uppermost in the leveller, and thus never meets a current of air which might contaminate it.

atmospheric contamination. In doing this, it is advisable to have an assistant to raise the glass covers. Tubes *b* and *c* are similarly treated,

and the resulting plates stacked in series on the top of *a*. The chamber is labelled and set aside for a few days till the colonies appear on the gelatin plates. The further procedure is of the same nature as with Petri's capsules.

3. *Esmarch's Roll Tubes*.—Here the principle is that of dilution as before. In each of three test-tubes  $1\frac{1}{4}$  or  $1\frac{1}{2}$  inch in diameter, gelatin to the depth of  $\frac{3}{4}$  of an inch is placed. These are sterilised. The gelatin is melted and inoculated in series with the bacterial mixture as in making plate cultures, but instead of being poured out it is rolled in a nearly horizontal position under a cold tap or on a block of ice till it solidifies as a uniformly thin layer on the inside of the tube. Practically we deal with a cylindrical sheet of gelatin instead of a flat one. A convenient form of tube for this method is one with a constriction a short distance below the plug of cotton wool (Fig. 20). The great disadvantage of the method is, that if organisms liquefying the gelatin be present, the liquefied gelatin contaminates the rest of the medium.



FIG. 20.  
Esmarch's tube  
for roll culture.

**Separation by Agar Media.**—1. *Agar Plates*.—The only difference between the technique here and that with gelatin depends on the difference in the melting-points of the two media. Agar, we have said, melts at  $98^{\circ}$  C., and becomes again solid a little under  $40^{\circ}$  C. As it is dangerous to expose organisms to a temperature much above  $42^{\circ}$  C., it is necessary in preparing tubes of agar to be used in plate cultures to first melt the agar, by boiling in a vessel of water for a few minutes, and then to cool them to about  $42^{\circ}$  C. before inoculating. The manipulation must be rapidly carried out, as the margin of time, before solidification occurs, is narrow; otherwise the details are the same as for gelatin. Esmarch's tubes are not suitable for use here, as the agar does not adhere well to the sides. If to the agar 2 per cent of a strong watery solution of pure gum arabic is added, Esmarch's tubes may, however, be used.

2. *Separation by Stroking Mixture on Surface of Agar Media*.—The bacterial mixture, instead of being mixed in the medium, is spread out on its surface. The method may be used both when the bacteria to be separated are in a fluid, and when contained in a fairly solid tissue or substance, such as a piece

of diphtheritic membrane. In the case of a tissue, for example, a small portion entangled in the loop of a platinum needle is stroked in successive parallel longitudinal strokes on sloped agar, the same aspect being brought in contact with the agar in all the strokes. Three strokes may be made on each tube, and three tubes are usually sufficient. In this process the organisms on the surface of the tissue are gradually rubbed off, and when growth has taken place it will be found that in the later strokes the colonies are less numerous than in the earlier, and sufficiently far apart to enable parts of them to be picked off without the needle touching any but one colony. When, as in the case of diphtheritic membrane, putrefactive organisms may be present on the surface of the tissue, these can be in great part removed by washing it well in cold water previously sterilised (*vide* Diphtheria). In the case of liquids, the loop is charged and similarly stroked. Tubes thus inoculated must be put in the incubator in the upright position and must be handled carefully so that the condensation water, which always is present in incubated agar tubes, may not run over the surface. Agar, poured out in a Petri's capsule and allowed to stand till firm, may be used instead of successive tubes. Here a sufficient number of strokes can be made in one capsule. Sloped blood-serum tubes may be used instead of agar. The method is rapid and easy, and gives good results.

**Separation of Pathogenic Bacteria by Inoculation of Animals.**—It is found difficult and often impossible to separate by ordinary plate methods certain pathogenic organisms, such as *b. tuberculosis*, *b. mallei*, and the pneumococcus, when such occur in conjunction with other bacteria. These grow best on special media, and the first two (especially the tubercle bacillus) grow so slowly that the other organisms present outgrow them, cover the whole plates, and make separation impossible. The method adopted in such cases is to inoculate an animal with the mixture of bacilli, wait until the particular disease develops, kill the animal, and with all aseptic precautions (*vide* p. 123) inoculate tubes of suitable media from characteristic lesions situated away from the seat of inoculation, *e.g.* from spleen in the case of *b. tuberculosis*, spleen or liver in the case of *b. mallei*, and heart blood in the case of pneumococcus.

**Separation by killing Non-spored Forms by Heat.**—This is a method which has a limited application. As has been said, the spores of a bacterium resist heat more than the vegetative forms. When a mixture contains spores of one bacterium and vegetative forms of this and other bacteria, then if the mixture

be boiled for a few minutes all the vegetative forms will be killed, while the spores will remain alive and will develop subsequently. This method can be easily tested in the case of cultivating *b. subtilis* from hay infusion. A little chopped-up hay is placed in a flask of water, which is boiled for about ten minutes. On this being allowed to cool and stand, in a day or two a scum forms on the surface, which is found to be a pure culture of the *bacillus subtilis*. The method is also often used to aid in the separation of *b. tetani*, *vide infra*.

### THE PRINCIPLES OF THE CULTURE OF ANAEROBIC ORGANISMS.

All ordinary media, after preparation, may contain traces of free oxygen, and will absorb more from the air on standing. (1) For the growth of anaerobes this oxygen may be expelled by the prolonged passing of an inert gas, such as hydrogen, through the medium (liquefied if necessary). Further, the medium must be kept in an atmosphere of the same gas, while growth is going on. (2) Media for anaerobes may be kept in contact with the air, if they contain a reducing agent which does not interfere with bacterial growth. Such an agent takes up any oxygen which may already be in the medium, and prevents further absorption. The reducing body used is generally glucose, though formate of sodium may be similarly employed. The preparation of such media has already been described (pp. 36, 37). In this case the medium ought to be of considerable thickness.

*The Supply of Hydrogen for Anaerobic Cultures.*—The gas is generated in a large Kipp's apparatus from pure sulphuric acid and pure zinc. It is passed through three wash-bottles, as in Fig. 21. In the first is placed a solution of lead acetate (1 in 10 of water) to remove any traces of sulphuretted hydrogen. In the second is placed a 1 in 10 solution of silver nitrate to remove any arseniatted hydrogen which may be present if the zinc is not quite pure. In the third is a 10 per cent solution of pyrogallie acid in caustic potash solution (1:10) to remove any traces of oxygen. The tube leading from the last bottle to the vessel containing the medium ought to be sterilised by passing through a Bunsen flame, and should have a small plug of cotton wool in it to filter the hydrogen germ-free.

**Separation of Anaerobic Organisms.**—(a) *By Roll-tubes.*—A 1½ inch test-tube has as much gelatin put into it as would be used in the Esmarch roll-tube method. It is corked with an india-rubber stopper having two tubes passing through it, as in Fig. 22. The ends of the tubes are partly drawn out as shown;



## 58 METHODS OF CULTIVATION OF BACTERIA

and covered with plugs of cotton wool. Three such test-tubes are prepared, and they are sterilised in the steam steriliser (p. 27). After sterilisation the gelatin is melted and one tube inoculated with the mixture containing the anaerobes; the second is inoculated from the first, and the third from the second, as in making ordinary gelatin plates. After inoculation the gelatin is kept liquid by the lower ends of the tubes being placed in water at about 30° C., and hydrogen is passed in through tube *x* for twenty minutes. The gas-supply tubes are then completely sealed off at *x* and *i*, and each test-tube is rolled as in Esmarch's method till the gelatin solidifies as a thin layer on the internal

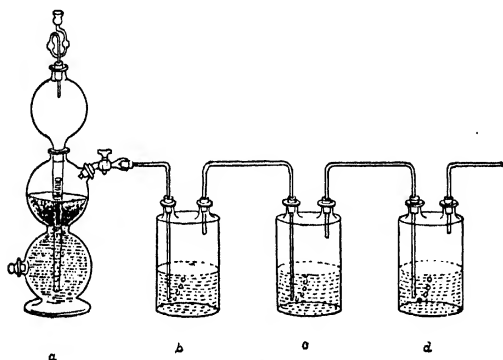


FIG. 21.—Apparatus for supplying hydrogen for anaerobic cultures.

*a.* Kipp's apparatus for manufacture of hydrogen. *b.* Wash-bottle containing 1-10 solution of lead acetate. *c.* Wash-bottle containing 1-10 solution of silver nitrate. *d.* Wash-bottle containing 1-10 solution of pyrogallous acid. (*b, c,* and *d* are intentionally drawn to a larger scale than *a* to show details.)

surface. A little hard paraffin may be run between the rim of the test-tube and the stopper, and round the perforations for the gas-supply tubes, to ensure that the apparatus is air-tight. The gelatin is thus in an atmosphere of hydrogen in which the colonies may develop. The latter may be examined and isolated in a way which will be presently described. The method is admirably suited for all anaerobes which grow at the ordinary temperature.

(*b*) **Bulloch's Apparatus for Anaerobic Culture.**—This can be recommended for plating out mixtures containing anaerobes, and for obtaining growths (especially surface growths) of the latter. It consists (Fig. 23) of a glass plate as base on which a bell jar can be firmly luted down with unguentum resinae. In

the upper part of the bell jar are two apertures furnished with ground stoppers, and through each of the latter passes a glass tube on which is a stop-cock. One tube, bent slightly just after passing through the stopper, extends nearly to the bottom of the chamber; the other terminates immediately below the stopper. In using the apparatus there is set on the base-plate a shallow dish, of slightly less diameter than that of the bell jar, and having a little heap of from two to four grammes of dry pyrogallic acid placed in it towards one side. Culture plates made in the usual way can be stacked on a frame of glass rods resting on the edges of the dish, or a beaker containing culture tubes can be placed in it. The bell jar is then placed in position so that the longer glass tube is situated over that part of the bottom of the shallow dish farthest away from the pyrogallic acid, and the bottom and stoppers are luted. The air in the bell jar is now expelled by passing a current of hydrogen through the short glass-tube,

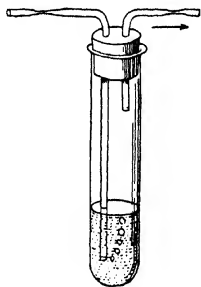


FIG. 22.—Esmarch's roll-tube adapted for culture containing anaerobes.

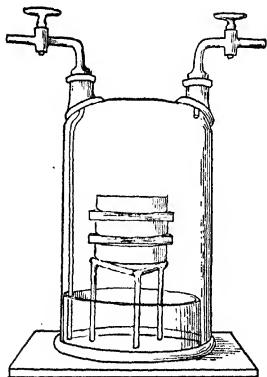


FIG. 23.—Bulloch's apparatus for anaerobic plate cultures.

and both stoppers are closed. A partial vacuum is then effected in the jar by connecting up the short tube with an air-pump, opening the tap, and giving a few strokes of the latter. A solution of 109 grms. solid caustic potash dissolved in 145 c.c. water is made, and into the vessel containing it a rubber tube connected with the long glass tube is made to dip, and the stopper of the latter being opened, the fluid is forced into the chamber and spreads over the bottom of the shallow dish; potassium pyrogallate is thus formed, which absorbs any free oxygen still present. Before the whole of the fluid is forced in,

the rubber tube is placed in a little boiled water, and this, passing through the glass tubes, washes out the potash and prevents

## 60 METHODS OF CULTIVATION OF BACTERIA

erosion of the glass. The whole apparatus may be placed in the incubator till growth occurs.

It is often advisable in dealing with material suspected to contain anaerobes to inoculate an ordinary deep glucose agar tube with it, and incubating for 24 or 48 hours, to then apply an anaerobic separation method to the resultant growth. Sometimes the high powers of resistance of spores to heat may be taken advantage of in aiding the separation (*vide* Tetanus).

**Cultures of Anaerobes.**—When by one or other of the above methods separate colonies have been obtained, growth may be maintained on media in contact with ordinary air. The media generally used are those which contain reducing agents, and the test-tubes containing the medium must be filled to a depth of 4 inches. They are sterilised as usual and are called “deep” tubes. The long straight platinum wire is used for inoculating, and it is plunged well down into the “deep” tube. A little air gets into the upper part of the needle track, and no growth takes place there, but in the lower part of the needle track growth occurs. From such “deep” cultures growths may be maintained indefinitely by successive sub-cultures in similar tubes. Even ordinary gelatin and agar can be used in the same way if the medium is heated to boiling-point before use to expel any absorbed oxygen.

*Carroll's Method for Anaerobic Cultures.*—This may be used with culture tubes containing any of the media suitable for anaerobes, with Esmarch's roll-tubes, or with fermentation tubes. There is required a dry tube of the same diameter as the culture tube, a short U-shaped glass tube, and two pieces of rubber tubing all of like diameter. The culture tube having been inoculated, the plug is pushed home below the lip of the tube. The ends of the U-tube are smeared with vaseline and a rubber tube slipped over each; the end of the culture tube being similarly treated, the free end of one of the rubber tubes is pushed over it till the glass of the U-tube is in contact with the glass of the culture tube. In the dry tube one or two grammes of pyrogallic acid are placed and the powder is packed down with a layer of filter paper. Ten or twenty cubic centimetres of a ten per cent solution of sodium hydrate are then poured in and the tube is quickly connected up by the rubber tubing with the other end of the U-tube. In this apparatus the oxygen is absorbed by the sodium pyrogallate and the conditions for anaerobic growth are fulfilled.

**Cultures of Anaerobes in Liquid Media.**—It is necessary to employ such in order to obtain the toxic products of the growth

of anaerobes. Glucose broth is most convenient. It is placed either (1) in a conical flask with a lateral opening and a perforated india-rubber stopper, through which a bent glass tube passes, as in Fig. 24, *a*, by which hydrogen may be delivered, or (2) in a conical flask with a rubber stopper furnished with two holes, as in Fig. 24, *b*, through a tube in one of which hydrogen is delivered, while through the tube in the other the gas escapes. The inner end of the gas delivery tube must in either case be below the surface of the liquid; the inner end of the lateral nozzle in the one case, and the inner end of the escape tube in the other, must of course be above the surface of the liquid. The single tube in the one case and the two tubes in the other

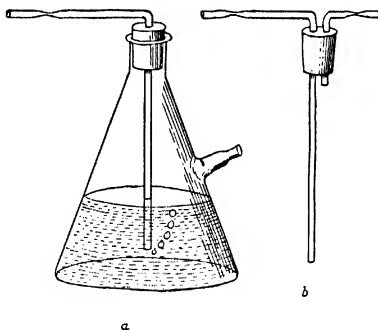


FIG. 24.

*a*. Flask for anaerobes in liquid media. Lateral nozzle and stopper fitted for hydrogen supply. *b*. A stopper arranged for a flask without lateral nozzle.

ought to be partially drawn out in a flame to facilitate subsequent complete sealing. The ends of the tubes through which the gas is to pass are previously protected by pieces of cotton wool tied on them. It is well previously to place in the tube, through which the hydrogen is to be delivered, a little plug of cotton wool. The flask being thus prepared, it is sterilised by methods B (2) or B (3). On cooling it is ready for inoculation. In the case of the flask with the lateral nozzle, the cotton-wool covering having been momentarily removed, a wire charged with the organism is passed down to the bouillon. In the other kind of flask the stopper must be removed for an instant to admit the wire. The flask is then connected with the hydrogen apparatus by means of a short piece of sterile india-rubber tubing, and hydrogen is passed through for half an hour. In the case of

## 62 METHODS OF CULTIVATION OF BACTERIA

flask (1), the lateral nozzle is plugged with molten paraffin covered with alternate layers of cotton wool and paraffin, the whole being tightly bound on with string. The entrance tube is now completely drawn off in the flame before being dis-

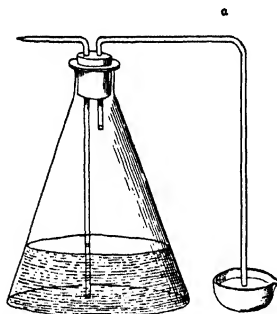


FIG. 25.—Flask arranged for culture of anaerobes which develop gas.

*b* is a trough of mercury into which exit tube dips.

connected from the hydrogen apparatus. In the case of flask (2), first the exit tube and then the entrance tube are sealed off in the flame before the flask is disconnected from the hydrogen apparatus. It is well in the case of both flasks to run some melted paraffin all over the rubber stopper. Sometimes much gas is evolved by anaerobes, and in dealing with an organism where this will occur, provision must be made for its escape. This is conveniently done by leading down the exit tube, and

letting the end just dip into a trough of mercury (Fig. 25), or into mercury in a little bottle tied on to the end of the exit tube. The pressure of gas within causes an escape at the mercury contact, which at the same time acts as an efficient valve. The method of culture in fluid media is used to obtain the soluble products of such anaerobes as the tetanus bacillus.

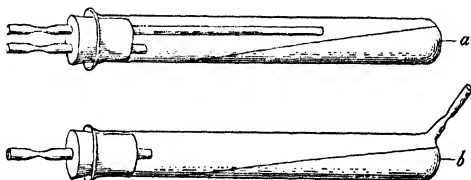


FIG. 26.—Tubes for anaerobic cultures on the surface of solid media.

When it is desired to grow anaerobes on the surface of a solid medium such as agar, tubes of the form shown in Fig. 26, *a* and *b*, may be used. A stroke culture having been made, the air is replaced by hydrogen as just described, and the tubes are fused at the constrictions. Such a method is of great value

when it is required to get the bacteria free from admixture of medium, as in the case of staining flagella.

#### MISCELLANEOUS METHODS.

**Hanging-drop Cultures.**—It is often necessary to observe micro-organisms alive, either to watch the method and rate of their multiplication, or to investigate whether or not they are motile. This is effected by making hanging-drop cultures. The

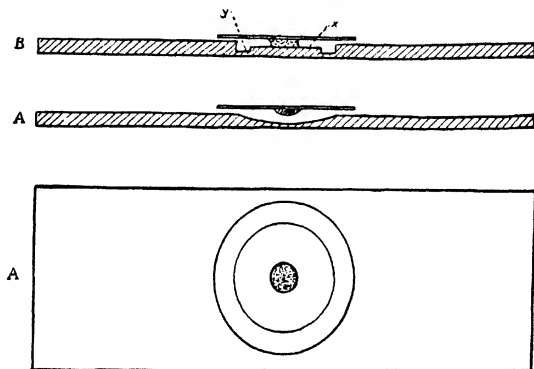


FIG. 27.

- A. Hollow-ground slide for hanging-drop cultures shown in plan and section.  
B. Another form of slide for similar cultures.

method in the form to be described is only suitable for aerobes. For this special slides are necessary. Two forms are in use and are shown in Fig. 27. In A there is ground out on one surface a hollow having a diameter of about half an inch. That shown in B explains itself. The slide to be used and a cover-glass are sterilised by hot air in a Petri's dish, or simply by being heated in a Bunsen and laid in a sterile Petri to cool. In the case of A, one or other of two manipulation methods may be employed. (1) If the organism be growing in a liquid culture, a loop of the liquid is placed on the middle of the under surface of the sterile cover-glass, which is held in forceps, the points of which have been sterilised in a Bunsen flame. If the organism be growing in a solid medium, a loopful of sterile bouillon is placed on the cover-glass in the same position, and a *very* small quantity of the culture (picked up with a platinum needle) is rubbed up in

## 64 METHODS OF CULTIVATION OF BACTERIA

the bouillon. The cover is then carefully lowered over the cell on the slide, the drop not being allowed to touch the wall or the edge of the cell. The edge of the cover-glass is covered with vaseline, and the preparation is then complete and may be placed under the microscope. If necessary, it may be first incubated and then examined on a warm stage. (2) The sterile cover-glass is placed on a sterile plate (an ordinary glass plate used for plate cultures is convenient). The drop is then placed on its *upper* surface, the details being the same as in the last case. The edge of the cell in the slide is then painted with vaseline, and the slide, held with the hollow surface downwards, is lowered on to the cover-glass, to the rim of which it of course adheres. The slide with the cover attached is then quickly turned right side up, and the preparation is complete.

In the case of B the drop of fluid is placed on the centre of the table *x*. The drop must be thick enough to come in contact with the cover-glass when the latter is lowered on the slide, and not large enough to run over into the surrounding trench *y*. The cover-glass is then lowered on to the drop, and vaseline is painted along the margin of the cover-glass. The method of microscopic examination is described on page 85.

*Anaerobic Hanging-drop Cultures.*—The growth and examination of bacteria in hanging-drops under anaerobic conditions involve consider-

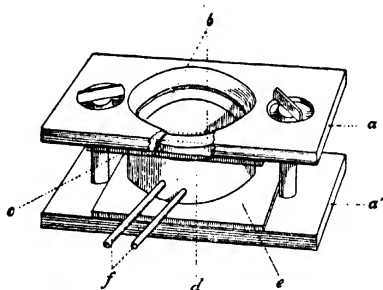


FIG. 28.—Graham Brown's chamber for anaerobic hanging-drops.

(A portion of one edge of upper plate is shown cut away.)

able difficulty, but may be carried out in an apparatus devised by Graham Brown (Fig. 28). It consists of two brass plates (*a* and *a'*) which can be approximated by screws, and which have rounded apertures in their middles  $\frac{1}{2}$  in. in diameter. These support two rubber rings, an upper thinner one (*b*) and a lower thick one (*d*), the inner diameters being the same as that of the apertures in the plates. Between

*b* and *d* is placed a stout cover-glass of suitable size (*c*) ; *d* is separated from the plate *a'* by a square plate of glass (*e*) (a portion of an ordinary glass-slide for microscopical purposes does well). Two small metal tubes (*f*) are inserted through the rubber *d*. Method of use:—Fix up the apparatus as shown above, the screws being just tight enough to keep the parts in position, and sterilise in the steam steriliser. Screw up more firmly so as to make the rubber bulge slightly. Fill a hypodermic syringe with some sterile glucose bouillon, push the needle through the rubber *d*, and, tilting the point of the needle against the glass *c*, slowly inject enough to form a drop on the under surface of *c*. Withdraw the syringe and inoculate its point with the bacterium, again introduce and inoculate the drop. Pass hydrogen through one of the tubes for fifteen minutes, close the ends of the tubes, and incubate at the required temperature. The apparatus can be put on the stage of a microscope and examined from time to time.

**The Counting of Colonies.**—An approximate estimate of the number of bacteria present in a given amount of a fluid (say, water) can be arrived at by counting the number of colonies which develop when that amount is added to a tube of suitable medium, and the latter plated and incubated. An ordinary plate should be used in such a case, and the medium poured out in as rectangular a shape as possible. For the counting, an apparatus such as is shown in Fig. 29 is employed.

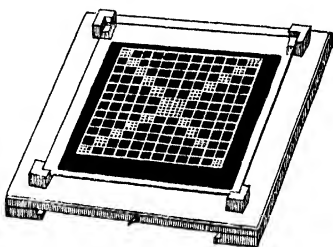


FIG. 29.—Apparatus for counting colonies.

This consists of a sheet of glass ruled into squares as indicated, and supported by its corners on wooden blocks. The table to which these blocks are attached has a dark surface. The plate-culture containing the colonies is laid on the top of the ruled glass. The numbers of colonies in, say, twenty of the smaller squares, are then counted, and an average struck. The total number of squares covered by the medium is then taken, and by a simple calculation the total number of colonies present can be obtained. Plate-cultures in Petri's dishes are sometimes employed for purposes of counting. The bottoms of such dishes are, however, never flat, and the thickness of the medium thus varies in different parts. If these dishes are to be used, a circle of the same size as the dish can be drawn with Chinese white on a black card, the circumference divided into equal arcs, and radii drawn. The



dish is then laid on the card, the number of colonies in a few of the sectors counted, and an average struck as before. In counting colonies it is always best to aid the eye with a small hand-lens.

**Method of counting Living Bacteria in a Culture.**—This is accomplished by putting into practice a dilution method such as that described on p. 52.

Measured amounts of high dilutions are plated, and the numbers of colonies which subsequently develop are counted. In applying such a

method it is necessary to have pipettes capable of measuring small quantities of fluid. Those discharging .05 and .1 c.c. will be found convenient, and such pipettes can have subdivisions which enable them to be used for measuring still smaller fractions of a cubic centimetre. Pipettes of this kind can be obtained at the instrument makers. Wright has described a method by which a pipette (Fig. 30) for measuring small quantities of fluid can be made from ordinary quill tubing. The method is as follows:—A piece of quill tubing about 15 cm. long is drawn out to a capillary stem. A standard 5 c.mm. pipette (such as that of the Gower's haemocytometer), or the pipette described later on p. 108, is filled with mercury and the metal transferred to the capillary stem and run down to near its extremity; the upper and lower limits of the mercury are marked with an oil pencil; the mercury is then displaced up the tube till its previously distal end is at the proximal

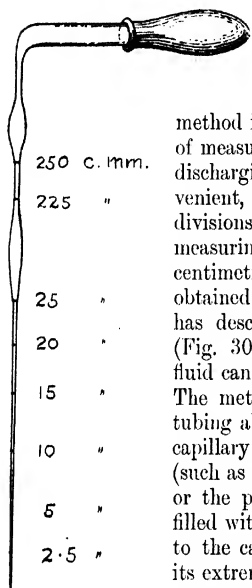


FIG. 30. — Wright's 250 c.mm. pipette fitted with nipple.

of the two marks, and a third mark is made at the new position of the upper end of the droplet; the manipulation is repeated three more times, and finally the tip of the tube beyond the lowest mark is broken off. Thus on the capillary part of the pipette we have five divisions, each capable of holding 5 c.mm. of fluid. The rest of the pipette is now calibrated so as to determine that part capable of containing 225 c.mm. and 250 c.mm. This is done by placing a rubber nipple on the wide end of the pipette and sucking up some water tinted with, say, methylene-blue till the 25 c.mm. mark is reached; a small air-bubble is then allowed to enter the

pipette, then other 25 c.mm. of fluid, then another bubble, and so on till nine volumes each of 25 c.mm. have been sucked up. A mark is then made on the tube at the upper level of this amount, other 25 c.mm. are sucked up, and another mark made. The fluid is expelled, the tube dried, and that part containing the 225 and 250 marks is drawn out into an almost capillary diameter, the manipulation by which the marks were originally arrived at is repeated, and thus in the new marks made a more accurate calibration for these amounts is attained. In order to form a safety chamber a second bulb is formed by drawing out the tube a little higher up as in the figure, and finally the upper inch or two are bent at right angles to the calibrated limb. In doing this a loop may be thrown on the plastic melted capillary tube exactly in the way in which a similar loop may be thrown on a piece of cord. With such a pipette any required dilution of a culture can be made on the principles already described.

**Wright's Method of counting the Bacteria in Dead Cultures.**—In the making of vaccines for use in Wright's procedures it is necessary to know the total number of bacterial cells, whether dead or living, present in a culture, for the dead as well as the living contain the toxins which may stimulate the therapeutic capacities of the body. The method consists in making a mixture of blood (whose content in red blood corpuscles is known) with the bacterial culture and comparing the number of bacteria with the number of corpuscles. The observer first estimates the red cells in his blood; a capillary pipette with a rubber nipple and with a mark near its capillary extremity is then taken, blood is sucked up to the mark, then an air-bubble, then (according to the empirical estimate the observer forms of the strength of his bacterial emulsion) either one volume of culture and three volumes of diluting fluid (*e.g.* .85 per cent sodium chloride) or two of culture and two of fluid, and so on; the five volumes are thoroughly mixed by being drawn backwards and forwards in the wide part of the pipette, a drop is then blown out on to a slide, and a blood film is spread which may be stained by Leishman's method. The bacteria and blood-corpuscles are now separately enumerated in a series of fields in different parts of the preparation. If a dilution has been taken in which a large number of bacteria are present, an artificial field may be used, made by drawing with the oil pencil a small square on a circular cover-glass and dropping the latter on to the diaphragm of the microscope eye-piece. Suppose, now, that the observer's blood contained 5,000,000 red cells per c.mm., that one volume of bacterial

emulsion and three of diluent had been present in the mixture, and that in the fields examined there were 500 red cells and 600 bacteria. It is evident that in the undiluted culture for 500 red cells there would have been 2400 bacteria. Now  $500:2400::5,000,000:24,000,000$ , which last figure is the number of bacteria per c.mm. of the emulsion.

**The Bacteriological Examination of the Blood.**—(a) This may be done by taking a small drop from the skin surface, *e.g.* the lobe of the ear. The part should be thoroughly washed with 1-1000 corrosive sublimate and dried with sterile cotton wool. It is then washed with absolute alcohol to remove the antiseptic, drying being allowed to take place by evaporation. A prick is then made with a sterile surgical needle; the drop of blood is caught with a sterile platinum loop and smeared on the surface of agar or blood serum. Film preparations for microscopic examination may be made at the same time. It is rare to obtain growths from the blood of the human subject by this method (*vide* special chapters), and if colonies appear the procedure should be repeated to exclude the possibility of accidental contamination.

(b) A larger quantity of blood may be obtained by puncture of a vein; this is the only satisfactory method, and should be the one followed whenever practicable. The skin over a vein in the forearm or on the dorsum of the foot having been sterilised, the vein is made turgid by pressure, and the needle of a syringe of 10-15 c.c. capacity, carefully sterilised, is then plunged obliquely through the skin into the lumen of the vessel. Several cubic centimetres of blood can thus be withdrawn into the syringe. Some of the blood (*e.g.* 1 c.c.) should be added to small flasks containing 50 c.c. of bouillon; the rest may be used for smearing the surface of agar tubes or may be added to melted agar at 42° C., which is then plated. The flasks, etc., are then incubated. By this method cultures can often be obtained where the former method fails, especially in severe conditions such as ulcerative endocarditis, streptococcus infection, etc. Part of the blood may be incubated by itself for twenty-four hours and cultures then made.

In examining the blood of the *spleen* a portion of the skin over the organ is sterilised in the same way, a few drops are withdrawn from the organ by a sterile hypodermic syringe and cultures made. (For microscopic methods, *vide* p. 87.)

**Bacteriological Examination of the Cerebro-spinal Fluid—Lumbar Puncture.**—This diagnostic procedure, which is sometimes called for in cases of meningitis, can be carried out with

a sterilised "antitoxin needle" as follows. The patient should lie on the right side, with knees somewhat drawn up and left shoulder tilted somewhat forward, so that the back is fully exposed. The skin over the lumbar region is then carefully sterilised, as above described, and the hands of the operator should be similarly treated. The spines of the lumbar vertebrae having been counted, the left thumb or forefinger is pressed into the space between the 3rd and 4th spines in the middle line; the needle is then inserted about half an inch to the right of the middle line at this level and pushed through the tissues, its course being directed slightly inwards and upwards, till it enters the subdural space. When this occurs fluid passes along the needle, sometimes actually spurting out, and should be received in a sterile test-tube. Several cubic centimetres of fluid can thus usually be obtained, no suction being required; thereafter it can be examined bacteriologically by the usual methods. The depth of the subdural space from the surface varies from a little over an inch in children to three inches, or even more, in adults—the length of the needle must be suited accordingly. In making the puncture it is convenient to have either a sterile syringe attached, or to have the thick end of the needle covered with a pad of sterile wool, which is of course removed at once when the fluid begins to flow. It is advisable to use the platinum needles which are specially made for the purpose, as a sudden movement of the patient may snap an ordinary steel needle.

**The Bacteriological Examination of Urine.**—In such an examination care must be taken to prevent the contamination of the urine by extraneous organisms. In the male it is usually sufficient to wash thoroughly the glans penis and the meatus with 1-1000 corrosive sublimate—the lips of the meatus being everted for more thorough cleansing. The urine is then passed into a series of sterile flasks, the first of which is rejected in case contamination has occurred. In the female, after similar precautions as regards external cleansing, the catheter must be used. The latter must be boiled for half an hour, and anointed with olive oil sterilised by half an hour's exposure in a plugged flask to a temperature of 120° C. Here, again, it is well to reject the urine first passed. It is often advisable to allow the urine to stand in a cool place for some hours, to then withdraw the lower portion with a sterile pipette, to centrifugalise this, and to use the urine in the lower parts of the centrifuge tubes for microscopic examination or culture.

**Filtration of Cultures.**—For many purposes it is necessary

## 70 METHODS OF CULTIVATION OF BACTERIA

to filter all the organisms from fluids in which they may have been growing. This is done especially in obtaining the soluble

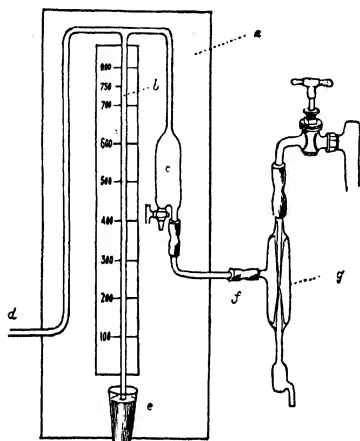


FIG. 31.—Geissler's vacuum pump arranged with manometer for filtering cultures. (The tap and pump are intentionally drawn to a larger scale than the manometer board to show details.)

toxic products of bacteria. The only filter capable of keeping back such minute bodies as bacteria is that formed from a tube of unglazed porcelain as introduced by Chamberland. The efficiency of such a filter depends on the fineness of the grain of the clay from which it is made; the finest is the Kitasato filter and the Chamberland "B" pattern; the next finest is the Chamberland "F" pattern, which is quite good enough for ordinary work. There are several filters, differing slightly in detail, all possessing the common principle. Sometimes the fluid is forced through the porcelain tube. In one form the filter consists practically of an ordinary tap screwed into the top of a porcelain tube. Through the latter the fluid is forced and passes into a chamber formed by a metal cylinder which surrounds the porcelain tube. The fluid escapes by an aperture at the bottom. Such a filter is very suitable for domestic use, or for use in surgical operating-theatres. As considerable pressure is necessary, it is evident it must be put on a pipe leading directly from the main. Sometimes, when fluids to be filtered are very albuminous, they are forced through a porcelain cylinder by compressed carbonic acid gas. For ordinary bacteriological work, filters of various kinds are in the market (such as those of Klein and

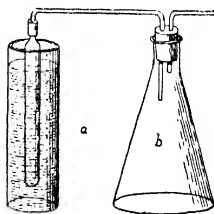


FIG. 32. Chamberland's candle and flask arranged for filtration.

others), but the most generally convenient is that in which the fluid is sucked through the porcelain by exhausting the air in the receptacle into which it is to flow. This is conveniently done by means of a Geissler's water-exhaust pump (Fig. 31, *g*), which must be fixed to a tap leading directly from the main. The connection with the tap must be effected by means of a piece of thick-walled rubber-tubing as short as possible, wired on to tap and pump, and firmly lashed externally with many turns of strong tape. Before lashing with the tape the tube may be strengthened by fixing round it with rubber solution strips of the rubbered canvas used for mending punctures

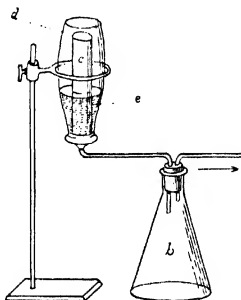


FIG. 33.—Chamberland's bougie arranged with lamp funnel for filtering a small quantity of fluid.



FIG. 34.—Bougie inserted through rubber stopper for same purpose as in Fig. 33.

in the outer case of a bicycle tyre. A manometer tube (*b*) and a receptacle (*c*) (the latter to catch any back flow of water from the pump if the filter accidentally breaks) are intercepted between the filter and the pump. These are usually arranged on a board *a*, as in Fig. 31. Between the tube *f* and the pump *g*, and between the tube *d* and the filter, it is convenient to insert lengths of flexible lead-tubing connected up at each end with short, stout-walled rubber-tubing.

Filters are arranged in various ways. (*a*) An apparatus is arranged as in Fig. 32. The fluid to be filtered is placed in the cylindrical vessel *a*. Into this a "candle" or "bougie" of porcelain dips. From the upper end of the bougie a glass tube with thick rubber connections, as in Fig. 32, proceeds to flask *b*

and passes through one of the two perforations with which the rubber stopper of the flask is furnished. Through the other opening a similar tube proceeds to the exhaust-pump. When the latter is put into action the fluid is sucked through the porcelain and passes over into flask *b*. This apparatus is very good, but not suitable for small quantities of fluid.

(b) A very good apparatus can be arranged with a lamp funnel and the porcelain bougie. These may be fitted up in two ways. (1) An india-rubber washer is placed round the bougie *c* at its glazed end (*vide* Fig. 33). On this the narrow end of the funnel *d*, which must, of course, be of an appropriate size, rests. A broad band of sheet rubber is then wrapped

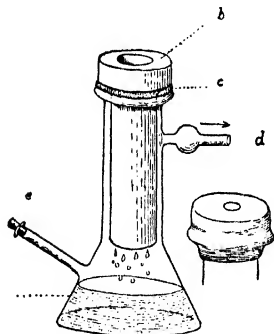


FIG. 35.—Muencke's modification of Chamberland's filter.

round the lower end of the funnel, and the projecting part of the bougie. It is firmly wired to the funnel above and to the bougie below. The extreme point of the latter is left exposed, and the whole apparatus, being supported on a stand, is connected by a glass tube with the lateral tube of the flask *b*; the tube *a* is connected with the exhaust-pump. The fluid to be filtered is placed between the funnel and the bougie in the space *e*, and is sucked through into the flask *b*.

(2) This modification is shown in Fig. 34. Into the narrow part of the funnel an india-rubber bung is fitted, with a perforation in it sufficiently large to receive the candle, which it should grasp tightly.

(c) Muencke's modification of the Chamberland filter is seen in Fig. 35. It consists of a thick-walled flask *a*, the lower part conical, the upper cylindrical, with a strong flange on the lip. There are two lateral tubes, one horizontal to connect with exhaust-pipe, and one sloping, by which the contents may be poured out. Passing into the upper cylindrical part of the flask is a hollow porcelain cylinder *b*, of less diameter than the cylindrical part of flask *a*. It is closed below, open above, and rests by a projecting rim on the flange of the flask, an asbestos washer, *c*, being interposed. The fluid to be filtered is placed

in the porcelain cylinder, and the whole top covered, as shown at *f*, with an india-rubber cap with a central perforation; the tube *d* is connected with the exhaust-pump and the tube *e* plugged with a rubber stopper.

When a large quantity of fluid is to be filtered, a receptacle such as that shown in Fig. 36 may be used. The tap in its bottom enables the filtrate to be removed without the apparatus being unshipped, but it is difficult

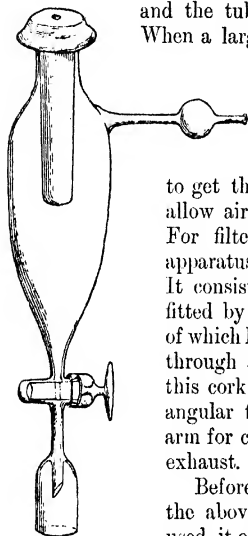


FIG. 36.—Flask fitted with porcelain bougie for filtering large quantities of fluid.

to get the tap to fit so accurately as not to allow air to pass into the vacuum chamber. For filtering small quantities of fluid the apparatus shown in Fig. 37 may be used. It consists of a small Chamberland bougie fitted by a rubber tube to a funnel, the stem of which has been passed through a rubber cork; this cork fits into a triangular flask with side arm for connection with exhaust.

Before any one of the above apparatus is used, it ought to be connected up as far as possible and sterilised in the Koch's steriliser. The ends of any important unconnected

parts ought to have pieces of cotton wool tied over them. After use the bougie is to be sterilised in the autoclave, and after being dried is to be passed carefully through a Bunsen flame, to burn off all organic matter. If the latter is allowed to accumulate the pores become filled up.

The success of filtration must be tested by inoculating tubes of media from the filtrate, and observing if growth takes place, as there may be minute perforations in the candles sufficiently large to allow bacteria to pass through. Filtered fluids keep for a long time if the openings of the glass vessels in which they are placed are kept

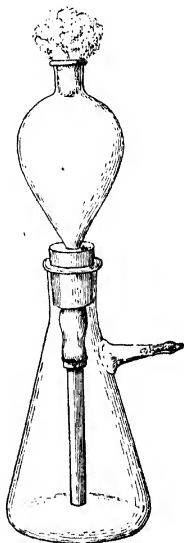


FIG. 37. — Flask for filtering small quantities of fluid.



## 74 METHODS OF CULTIVATION OF BACTERIA

thoroughly closed, and if these vessels be kept in a cool place in the dark. A layer of sterile toluol about half an inch thick ought to be run on to the top of the filtered fluid to protect the latter from the atmospheric oxygen.

Instead of being filtered off, the bacteria may be killed by various antiseptics, chiefly volatile oils, such as oil of mustard (Roux). These oils are stated to have no injurious effect on the chemical substances in the fluid, and they may be subsequently removed by evaporation. It is not practicable to kill the bacteria by heat when their soluble products are to be studied, as many of the latter are destroyed by a lower temperature than is required to kill the bacteria themselves.

Bacteria can be almost entirely removed from fluid cultures by spinning the latter in a centrifuge of very high speed (*e.g.* C. J. Martin's turbine centrifuge), and this method is sometimes adopted in practice.

### **The Observation of Bacterial Fermentation of Sugars, etc.**

—The capacity of certain species of bacteria to originate fermentations in sugars constitutes an important biological factor. It is well to consider this factor in relation to the chemical constitution of the sugars. These bodies are now known to be (to use the definition of Holleman) aldehyde or ketone alcohols containing one or more hydroxyl groups, one of which is directly linked to a carbon atom in union with carbonyl. The group characteristic of a sugar is thus  $\text{—CHOH—CO—}$ . The sugars are divided into monosaccharides or monoses, disaccharides (dioses), and polysaccharides (polyoses). The members of the last two groups may be looked on as derived from the combination of two or more molecules of a monosaccharide with the elimination of water (*e.g.*  $2\text{C}_6\text{H}_{12}\text{O}_6 = \text{C}_{12}\text{H}_{22}\text{O}_{11} + \text{H}_2\text{O}$ ).

*Monosaccharides.*—These are classified according to the number of C atoms they contain. The pentoses ordinarily used are arabinose (obtained from gum arabic), rhamnose and xylose (from wood). Among the hexoses are glucose (dextrose) with dextro-rotatory properties. Glucose is an aldehyde alcohol (aldose). In fruit there is also a ketone alcohol (ketose) called fructose, which from its lævo-rotatory properties is also known as lævulose. Other hexoses are mannose (from the vegetable ivory nut) and galactose (a hydrolytic derivative of lactose).

*Disaccharides* ( $\text{C}_{12}\text{H}_{22}\text{O}_{11}$ ).—The ordinary members of this group are maltose (derived from starch), lactose, and cane sugar (sucrose, saccharose).

*Polysaccharides.*—Examples are starch, raffinose, inulin (from dahlia roots), dextrin, arabin, glycogen, cellulose.

If we consider sugars generally from the point of view of the capacity of yeast to originate alcoholic fermentation in them, we may say that the simpler the constitution of the sugar the more easily is it fermented. Thus the monosaccharides are more easily acted on by yeast than the di- or polysaccharides. Usually an independent process resulting in the splitting of the higher into the lower is preliminary to the alcoholic fermentation. Thus yeast first inverts cane sugar into glucose and fructose and then acts on these products. From what is known it is probable that similar facts hold with regard to the action of bacteria.

Besides sugars other alcohols with large molecules may be broken down by bacterial action, and these bodies have been used for differentiating the properties of allied bacteria. Among these substances may be mentioned the trihydric alcohol glycerol (glycerin), the tetrahydric erythritol and the hexahydric dulcitol (dulcite), mannitol (mannite), and sorbitol (sorbite).

Similarly certain glucosides, such as salicin, coniferin, etc., have been used for testing the fermentative properties of bacteria. Other substances allied to sugars (*e.g.* inosite) have also been used.

The end products of bacterial fermentations may be various. They differ according to the sugar employed and according to the species of bacterium under observation, and frequently a species which will ferment one sugar has no effect on another. The substances finally produced, speaking roughly, may be alcohols, acids, or gaseous bodies (chiefly carbon dioxide, hydrogen, and methane). For the estimation of the first groups complicated chemical procedure may be necessary. The tests usually employed for the detection of ordinary fermentative processes depend on two kinds of changes, namely (*a*) the evolution of gases and (*b*) the formation of acids. Generally speaking, we may say that such tests are reliable and the methods to be pursued are simple. Besides such gases as those named some organisms give rise to sulphuretted hydrogen by breaking up the proteid. The formation of this gas can be detected by the blackening of lead acetate when it is added to the gas-containing medium.

In testing the effect of a bacterium on a given sugar it is essential that this sugar alone be present; the basis of the medium ought therefore to be either peptone solution (*v. p.* 38) or a dextrose-free bouillon (*v. infra*). The sugar or other substance is added in the proportion of from a half to one per cent, and care is taken not to overheat during sterilisation.

To obtain a "dextrose-free" bouillon it is usual to inoculate ordinary bouillon with some organism, such as *b. coli*, which is known to ferment

dextrose, and allow the latter to act for forty-eight hours. The bouillon is then filtered and re-sterilised. A sample is tested for another period of forty-eight hours with *b. coli*, to make certain that all the dextrose has been removed. If no fresh gas-formation is observed, then to the remainder of the bouillon the sugar to be investigated may be added. It is preferable that the addition should be made in the form of a sterile solution. If the sugar in solid form be placed in the bouillon and this then sterilised, there is danger that chemical changes may take place in the sugar, in consequence of its being heated in the presence of substances (such as the alkali) which may act deleteriously upon it; in any case sterilisation should not be at a temperature above 100° C.

For the observation of gas-formation either of the following methods may be employed:—

(1) *Durham's Tubes* (Fig. 38, *b*).—The plug of a tube which contains about one-third more than usual of a liquid medium is

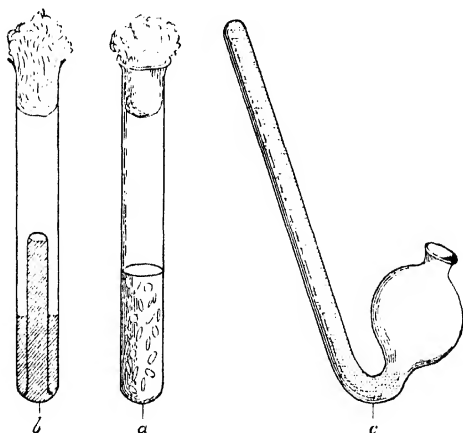


FIG. 38.—Tubes for demonstrating gas-formation by bacteria.

*a*, tube with "shake" culture.  
*b*, Durham's fermentation tube.  
*c*, ordinary form of fermentation tube.

removed, and a small test-tube is slipped into the latter mouth downwards. The plug is replaced and the tube sterilised thrice for ten minutes at 100° C. The air remaining in the smaller tube is thereby expelled. The tube is then inoculated with the bacterium to be tested. Any gas developed collects in the upper part of the inner tube.

(2) *The Fermentation Tube* (Fig. 38, *c*).—This consists of a tube of the form shown, and the figure also indicates the extent

to which it ought to be filled. It is inoculated in the bend with the gas-forming organism, and when growth occurs the gas collects in the upper part of the closed limit, the medium being displaced into the bulb.

For the observation of the effect of an organism on glucose the following method may be employed:—

*Gelatin Shake Cultures* (Fig. 38, *a*).—The gelatin in the tube is melted as for making plates; while liquid it is inoculated with the growth to be observed, and shaken to distribute the organisms throughout the jelly. It is then allowed to solidify, and is set aside at a suitable temperature. If the bacterium used is a gas-forming one, then, as growth occurs, little bubbles appear round the colonies.

In this method the gas-formation results from fermentation of the glucose naturally present in the medium from transformation of the glycogen of muscle. The amount of glucose naturally present, however, varies much, and therefore glucose should be added to the medium if the effects on this sugar are to be observed with certainty. The shake culture method may be utilised for observing fermentation in other sugars by adding to peptone solution containing the sugar 10-15 per cent of gelatin.

The *development of an acid reaction* is demonstrated by the addition of an indicator to the medium, litmus being generally used. The details of composition of such media have already been given. In Hiss's serum water media the production of acid also leads to coagulation of the medium. Sometimes acid is formed very slowly from sugars, so that it is well to keep the cultures under observation for several days.

Acid and gas-formation may be simultaneously tested for, by placing the fluid medium containing the indicator in Durham's tubes.

In all tests in which sugars are used a control uninoculated tube ought to be incubated with the bacterial cultures, as changes in reaction sometimes spontaneously occur in media containing unstable sugars.

The capacity of an organism to produce acid may be measured by taking a standard amount of a fluid medium and allowing growth to take place for a standard time, and then adding an amount of, say, decinormal soda solution sufficient to bring the litmus back to the tint of the original medium.

**The Observation of Indol-formation by Bacteria.**—The formation of indol from albumin by a bacterium sometimes constitutes an important specific characteristic. To observe indol

## 78 METHODS OF CULTIVATION OF BACTERIA

production the bacterium is grown, preferably at incubation temperature, in a fluid medium containing peptone. The latter may either be ordinary bouillon or preferably peptone solution (see p. 38). Indol production is recognised by the fact that when acted on by nitric acid *in the presence of nitrites*, a nitroso-indol compound is produced, which has a rosy red colour. Some bacteria (*e.g.* the cholera vibrio) produce nitrites as well as indol, but usually in making the test (*e.g.* in the case of *b. coli*) the nitrites must be added. This is effected by adding to an ordinary tube of medium 1 c.c. of a .02 per cent solution of potassium nitrite, and testing with pure nitric or sulphuric acid. In any case only a drop of the acid need be added to, say, 10 c.c. of medium. If no result be obtained at once it is well to allow the tube to stand for an hour, as sometimes the reaction is very slowly produced. In many instances incubation at 37° C. for several days may be necessary before the presence of indol is demonstrable. The amount of indol produced by a bacterium seems to vary very much with certain unknown qualities of the peptone. It is well therefore to test a series of peptones with an organism (such as the *b. coli*) known to produce indol, and noting the sample with which the best reaction is obtained, to reserve it for making media to be used for the detection of this product.

**The Drying of Substances in vacuo.**—As many substances, for example toxins and antitoxins, with which bacteriology is concerned would be destroyed by drying with heat as is done in ordinary chemical work, it is necessary to remove the water at the ordinary room temperature. This is most quickly effected by drying *in vacuo* in the presence of some substance such as strong sulphuric acid which readily takes up water vapour. The vacuum produced by a water-pump is here not available, as in such a vacuum there must always be water vapour present. An air-pump is therefore to be employed. Here we have found the Geryk pump most efficient, and it has this further advantage, that its internal parts are lubricated with an oil of very low vapour density so that almost a perfect vacuum is obtainable. The apparatus is shown in Fig. 39. The vacuum chamber consists of a bell-jar set on a brass plate. A perforation in the centre of the latter leads into the pipe *a*, which can be connected by strong-walled rubber-tubing with the air-pump, and which can be cut off from the latter by a stop-cock *b*. In using the apparatus the substance to be dried is poured out in flat dishes (one-half of a Petri capsule does very well), and these are stacked alternately with similar dishes of strong sulphuric acid on a

stand which rests on the brass plate. The edge of the bell-jar is well luted with unguentum resinæ and placed in position and the chamber exhausted. In a few hours, if, as is always advisable, each dish have contained only a thin layer of fluid, the drying will be complete. The vacuum is then broken by admitting air very slowly through a bye-pass *c*, and the bell-jar is removed. In such an apparatus it is always advisable, as is shown in the figure, to have interposed between the pump and the vacuum chamber a Wolff's bottle containing sulphuric acid. This protects the oil of the pump from contamination with

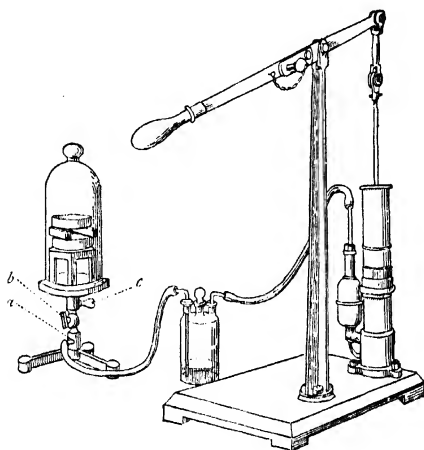


FIG. 39.—Geryk air-pump for drying *in vacuo*.

water vapour. Whenever the vacuum is produced the rubber-tube should be at once disconnected from *a*, the cock *b* being shut. It is advisable when the apparatus is exhausted to cover the vacuum chamber and the Wolff's bottle with wire guards covered with strong cloth, in case, under the external pressure, the glass vessels give way.

**The Storing and Incubation of Cultures.**—Gelatin cultures must be grown at a temperature below their melting-point, *i.e.* for 10 per cent gelatin, below 22° C. They are usually kept in ordinary rooms, which vary, of course, in temperature at different times, but which have usually a range of from about 12° C. to 18° C. Agar and serum media are usually employed to grow bacteria at a higher temperature, corresponding to that at which

the organisms grow best, usually 37° C. in the case of pathogenic organisms. For the purpose of maintaining a uniform temperature incubators are used. These vary much in the details of their structure, but all consist of a chamber with double walls between which some fluid (water or glycerin and water) is placed, which, when raised to a certain temperature, ensures a fairly constant distribution of the heat round the chamber. The latter is also furnished with double doors, the inner being usually of glass. Heat is supplied from a burner fixed below. These burners vary much in design. Sometimes a mechanism devised in Koch's laboratory is affixed, which automatically turns off the gas if the light be accidentally extinguished. Between the tap supplying the gas, and the burner, is interposed a gas regulator. Such regulators vary in design, but for ordinary chambers which require to be kept at a constant temperature, Reichert's is as good and simple as any and is not expensive. It is shown in Fig. 40.

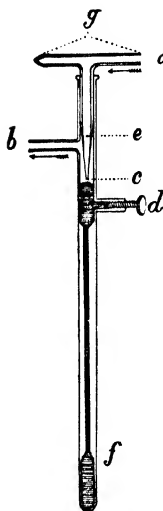


FIG. 40.—Reichert's gas regulator.

It consists of a long tube *f* closed at the lower end, open at the upper, and furnished with two lateral tubes. The lower part is filled with mercury up to a point above the level of the lower lateral tube. The end of the latter is closed by a brass cap through which a screw *d* passes, the inner end of which lies free in the mercury. The height of the latter in the perpendicular tube can thus be varied by increasing or decreasing the capacity of the lateral tube by turning the screw a few turns out of or into it. Into the upper open end of the perpendicular tube fits accurately a bent tube *g*, drawn out below to a comparatively small open point *c*, and having in its side a little above the point a minute needle-hole

called the peephole or bye-pass *e*. To fix the apparatus the long mercury bulb is placed in the jacket of the chamber to be controlled, tube *a* is connected to gas supply, tube *b* with the burner. The upper level of the mercury should be some distance below the lower open end of tube *c*. The burner is now lit. The gas passes in at *a* through *c* and *e* and out at *b* to the burner. When the thermometer in the interior of the chamber indicates that the desired temperature has been reached, the screw *d* is turned till the mercury reaches the end of the tube *c*. Gas can only now pass through the peephole *e*, and the flame goes down. The contents of the jacket cool, the mercury contracts off the end of tube *c*, and the flame rises. This alternation going on, the temperature of the chamber is kept very nearly constant. If the mercury cuts off the gas supply before the desired temperature is reached, and

the screw *d* is as far out as it will go, then some of the mercury must be removed. Similarly, if when the desired temperature is reached and the screw *d* is as far in as it can go, the mercury does not reach *c*, some more must be introduced. If the amount of gas which passes through the peephole is sufficient still to raise the temperature of the chamber when *c* is closed by the rise of the mercury, then the peephole is too large. Tube *c* must be unshipped and *e* plastered over with sealing-wax, which is pricked, while still soft, with a very fine needle. The gas flame, when only the peephole is supplying gas, ought to be sufficiently large not to be blown out by small currents of air. If the pressure of gas supplied to a regulator varies much in the 24 hours a pressure regulator ought to be interposed between the gas tap and the instrument. Several varieties of these can be obtained. In all cases *g* ought to be fixed to *b* with a turn of wire.

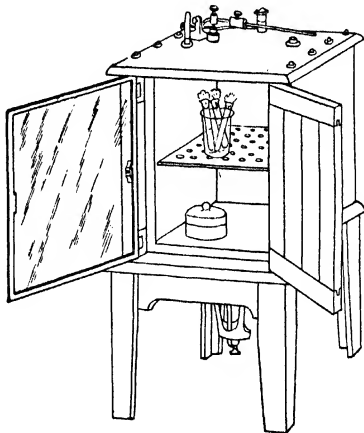


FIG. 41.—Hearson's incubator for use at 37° C.

The varieties of incubators are, as we have said, numerous. The most complicated and expensive are made by German manufacturers. Many of these are unsatisfactory. They easily get out of order and are difficult to repair. We have found those of Hearson of London extremely good, and in proportion to their size much cheaper than the German articles. They are fitted with an admirable regulator. It is preferable in using an incubator to connect the regulator with the gas supply and with the Bunsen by flexible metal tubing. It is necessary to see that there is not too much evaporation from the surface of cultures placed within incubators, otherwise they may quickly dry up. It is thus advisable to raise the amount of water vapour in the interior by having in the bottom of the incubator a flat dish full



of water from which evaporation may take place. Tubes which will require to be long in the incubator should have their plugs covered either by india-rubber caps or by pieces of sheet rubber tied over them. These caps should be previously sterilised in 1-1000 corrosive sublimate and then dried. Before they are placed on the tubes the cotton-wool plug ought to be well singed in a flame. "Cool" incubators are often used for incubating gelatin at  $21^{\circ}$  to  $22^{\circ}$  C. An incubator of this kind fitted with a low-temperature Hearson's regulator is in the market.

**Method of mounting Bacterial Cultures as Permanent Museum Specimens.** (Richard Muir).—(a) *Stab or stroke cultures in nutrient gelatin or agar media.*—When the culture shows typical characters, further growth is arrested by placing tube in a formol vapour chamber, or by saturating the cotton-wool plug with strong formalin. Then leave for a day or two. Make up following:—

|  |           |
|--|-----------|
| (1) Thymol Water (saturated in cold) . . . . . | 100 c.c.  |
| Glycerin . . . . .                             | 20 c.c.   |
| Acetate of Potash . . . . .                    | 5 grams.  |
| Coignet's (gold label) Gelatin . . . . .       | 10 grams. |

Render the mixture acid to litmus with acetic acid ; clear with white of egg and filter.

Warm to about  $40^{\circ}$  C., and removing cotton-wool plug from culture take a little of the preserving fluid in a pipette and allow to run gently over surface of medium in tube. Place in such a position that a thin layer of the preserving medium remains completely covering the growth and the surface of culture medium. The gelatin is now allowed to solidify. Add three or four drops of strong formalin to the tube and fill up to within a quarter of an inch of the top of the tube with the following fluid:—

|  |          |
|--|----------|
| (2) Thymol Water (saturated in cold) . . . . . | 100 c.c. |
| Glycerin . . . . .                             | 20 c.c.  |
| Acetate of Potash . . . . .                    | 5 grams. |

Cover top of tube with a small piece of paper so as to keep out dust, allow to stand for a day or two so that small air-bells may rise to the surface.

To seal tube, pour melted paraffin gently on to the surface of fluid to near the top of tube ; allow to solidify. Cover paraffin with layer of alcoholic orange shellac cement ; allow this to set and repeat until the cement becomes level with top of test tube. When set, a few drops of black lacquer are put on and a circular cover-glass of about the same diameter as the mouth of tube is placed so as completely to seal it.

(b) The following method is useful for preserving *plate cultures*. Instead of making the cultures in Petri's capsules, use ordinary watch-glasses. The watch-glass is sterilised in a Petri's capsule, and the inoculated medium is poured out into the watch-glass, allowed to solidify in the usual way, and left in the Petri's capsule until the colonies of growth have developed. The watch-glass is now removed from capsule and a layer of the preserving gelatin medium (1), to which have been added a few drops of strong formalin, is allowed to spread over the surface of the culture medium. When the layer is solidified the watch-glass is filled up with the same, and a clean square or oblong piece of glass (which of course should be of slightly larger diameter than the watch-glass) is now carefully placed over watch-glass, care being taken that no air-bells are formed. The edge of watch-glass should be closely applied to the glass cover and left in position until the gelatin has solidified. The superfluous gelatin is now removed, and the glasses sealed first with the orange shellac cement, then with black lacquer. It is now finished off by using a circular mask of suitable size.

The various kinds of solid media used in the cultivation of bacteria, such as blood serum, potato, bread paste, etc., can be treated in the same manner with excellent results.

**General Laboratory Rules.**—On the working bench of every bacteriologist there should be a large dish of 1-1000 solution of mercuric chloride in water. Into this all tubes, vessels, plates, hanging-drop cultures, etc., which have contained bacteria and with which he has finished, ought to be at once plunged (in the case of tubes the tube and plug should be put in separately). On no account whatever are such infected articles to be left lying about the laboratory. The basin is to be repeatedly cleaned out. All the glass is carefully washed in repeated changes of tap water to remove the last trace of perchloride of mercury, a very minute quantity of which is sufficient to inhibit growth. Old cultures which have been stored for a time and from which fresh sub-cultures have been made ought to be steamed in the Koch's steriliser for two or three hours, or in the autoclave for a shorter period, and the tubes thoroughly washed out. Besides a basin of mercuric chloride solution for infected apparatus, etc., there ought to be a second reserved for the worker's hands in case of any accidental contamination. When, as in public-health work, a large number of tubes are being daily put out of use, they may be placed in an enamelled slop-pail and this when full is placed in the steam steriliser.

A white glazed tile on which a bell-jar can be set is very

## 84 METHODS OF CULTIVATION OF BACTERIA

convenient to have on a bench. Infective material in watch-glasses can be placed thus under cover while investigation is going on, and if anything is spilled the whole can be easily disinfected. In making examinations of organs containing virulent bacteria, the hands should be previously dipped in 1-1000 mercuric chloride and allowed to remain wet with this solution. No food ought to be partaken of in the laboratory, and pipes, etc., are not to be laid with their mouth-pieces on the bench. No label is to be licked with the tongue. Before leaving the laboratory the bacteriologist ought to wash the hands and forearms with 1-1000 mercuric chloride and then with yellow soap. In the case of any fluid containing bacteria being accidentally spilt on the bench or floor, 1-1000 mercuric chloride is to be at once poured on the spot. The air of the laboratory ought to be kept as quiet as possible.

## CHAPTER III.

### MICROSCOPIC METHODS—GENERAL BACTERIOLOGICAL DIAGNOSIS—INOCULATION OF ANIMALS.

**The Microscope.**—For ordinary bacteriological work a good microscope is essential. It ought to have a heavy stand, with rack and pinion and fine adjustment, a double mirror (flat on one side, concave on the other), a good condenser, with an iris diaphragm, and a triple nose-piece. It is best to have three objectives, either Zeiss A, D, and  $\frac{1}{2}$ -inch oil immersion, or the lenses of other makers corresponding to these. The oil immersion lens is essential. It is well to have two eye-pieces, say Nos. 2 and 4 of Zeiss or lenses of corresponding strengths. The student must be thoroughly familiar with the focussing of the light on the lens by means of the condenser, and also with the use of the immersion lens. It may here be remarked that when it is desired to bring out in sharp relief the margins of unstained objects, *e.g.* living bacteria in a fluid, a narrow aperture of the diaphragm should be used, whereas, in the case of stained bacteria, when a pure colour picture is desired, the diaphragm ought to be widely opened. The flat side of the mirror ought to be used along with the condenser. When the observer has finished for the time being with the immersion lens he ought to wipe off the oil with a piece of silk or very fine washed linen. If the oil has dried on the lens it may be moistened with xylol—never with alcohol, which will dissolve the material by which the lens is fixed in its metal carrier.

**Microscopic Examination of Bacteria.** 1. **Hanging-drop Preparations.**—Micro-organisms may be examined: (1) alive or dead in fluids; (2) in film preparations; (3) in sections of tissues. In the two last cases advantage is always taken of the affinity of bacteria for certain stains. When they are to be examined in fluids a drop of the liquid may be placed on a slide

and covered with a cover-glass.<sup>1</sup> It is more usual, however, to employ hanging-drop preparations. The technique of making these has already been described (p. 63). In examining them microscopically, it is necessary to use a very small diaphragm. It is best to focus the edge of the drop with a low-power objective, and, arranging the slide so that part of the edge crosses the centre of the field, to clamp the preparation in this position. A high-power lens is then turned into position and lowered by the coarse adjustment to a short distance above its focal distance; it is now carefully screwed down by the fine adjustment, the eye being kept at the tube meanwhile. The shadow of the edge will be first recognised, and then the bacteria must be carefully looked for. Often a dry lens is sufficient, but for some purposes the oil immersion is required. If the bacteria are small and motile a beginner may have great difficulty in seeing them, and it is well to practise at first on some large non-motile form such as anthrax. In fluid preparations the natural appearance of bacteria may be studied, and their rate of growth determined. The great use of such preparations, however, is to find whether or not the bacteria are motile, and for determining this point it is advisable to use either broth or agar cultures not more than twenty-four hours old. In the latter case a small fragment of growth is broken down in broth or in sterile water. Sometimes it is an advantage to colour the solution in which the hanging-drop is made up with a minute quantity of an aniline dye, say a small crystal of gentian violet to 100 c.c. of bouillon. Such a degree of dilution will not have any effect on the vitality of the bacteria. Ordinarily, living bacteria will not take up a stain, but even though they do not, the contrast between the unstained bacteria and the tinted fluid will enable the observer more easily to recognise them.

**2. Film Preparations.** (*a*) *Dry Method.*—This is the most extensively applicable method of microscopically examining bacteria. Fluids containing bacteria, such as blood, pus, scrapings of organs, can be thus investigated, as also cultures in fluid and solid media. The first requisite is a perfectly clean cover-glass. Many methods are recommended for obtaining such. The test of this being accomplished is that, when the drop of fluid containing the bacteria is placed upon the glass, it can be uniformly spread with the platinum needle all over the surface without showing any tendency to retract into droplets.

<sup>1</sup> In bacteriological work it is essential that cover-glasses of No. 1 thickness (*i.e.* .14 mm. thick) should be used, as those of greater thickness are not suitable for a  $\frac{1}{2}$ -in. lens.

The best method is that recommended by Van Ermengem. The cover-glasses are placed for some time in a mixture of concentrated sulphuric acid 6 parts, potassium bichromate 6 parts, water 100 parts, then washed thoroughly in water and stored in absolute alcohol. For use, a cover-glass is either dried by wiping with a clean duster or is simply allowed to dry. This method will amply repay the trouble, and really saves time in the end. A clean cover having been obtained, the film preparation can now be made. If a fluid is to be examined a loopful may be placed on the cover-glass, and either spread out over the surface with the needle, or another clean cover may be placed on the top of the first, the drop thus spread out between them and the two then drawn apart. When a culture on a solid medium is to be examined a loopful of distilled water is placed on the cover-glass and a minute particle of growth rubbed up in it and spread over the glass. The great mistake made by beginners is to take too much of the growth. The point of the



FIG. 42.—Cornet's forceps for holding cover-glasses.

straight needle should just touch the surface of the culture, and when this is rubbed up in the droplet of water and the film dried, there should be an opaque cloud just visible on the cover-glass. When the film has been spread it must next be dried by being waved backwards and forwards at arm's-length above a Bunsen flame. The film must then be fixed on the glass by being passed three or four times slowly through the flame. In doing this a good plan is to hold the cover-glass between the right forefinger and thumb; if the fingers just escape being burned no harm will accrue to the bacteria in the film.

In making films of a thick fluid such as *pus* it is best to spread it out on one cover with the needle. The result will be a film of irregular thickness, but sufficiently thin at many parts for proper examination. Scrapings of organs may be smeared directly on the cover-glasses.

In the case of *blood*, a fairly large drop should be allowed to spread itself between two clean cover-glasses, which are then to be slipped apart, and being held between the forefinger and thumb are to be dried by a rapid to-and-fro movement in the air. A film prepared in this way may be too thick at one edge, but at the other is beautifully thin. If it is desired to preserve the red blood corpuscles in such a film it may be fixed by one of the following methods: by being placed (a) in a hot-air

chamber at 120° C. for half an hour, (b) in a mixture of equal parts of alcohol and ether for half an hour, then washed and dried, (c) in formol-alcohol (Gulland) (formalin 1 part, absolute alcohol 9 parts) for five minutes, then washed and dried, or (d) in a saturated solution of corrosive sublimate for two or three minutes, then washed well in running water and dried. (Fig. 71 shows a film prepared by the last method.) In using the Romanowsky stains no previous fixation is necessary (*vide infra*). In the case of *urine*, the specimen must be allowed to stand, and films made from any deposit which occurs; or, what is still better, the urine is centrifugalised, and films made from the deposit which forms. After dried films are thus made from urine it is an advantage to place a drop of distilled water on the film and heat gently to dissolve the deposit of salts; then wash in water and dry. In this way a much clearer picture is obtained when the preparation is stained.

Within recent years it has become common to make blood films on ordinary microscopic slides instead of upon cover-glasses. Here the slides must be clean. This can be effected by washing thoroughly first with weak alkali and then with water and storing in alcohol. For use, a slide is taken from the alcohol and the fluid adhering to it set on fire and allowed to burn off, a dry clean slide being thus obtained. To make a film on such, a small drop of blood is placed near one end, the edge of a second clean slide is lowered through the drop on to the surface of the glass on which the blood has been placed. This second slide is held at an angle to the first, on which it rests by its edge. The droplet of blood by capillarity spreads itself in the angle between the two slides. The edge of the second slide is then stroked along the surface of the first slide, and in this procedure the blood is spread out in a film whose thickness can be regulated by the angle formed by the second slide. Large-sized films can thus be obtained, and when these are stained they are often examined without any cover-glass being placed upon them. A drop of cedar oil is placed on the preparation, and after use this can be removed by the careful application of xylol.

Films dried and fixed by the above methods are now ready to be stained by the methods to be described below.

(b) *Wet Method*.—If it is desired to examine the fine histological structure of the cells of a discharge as well as to investigate the bacteria present, it is advisable to substitute “wet” films for the “dried” films, the preparation of which has been described. The nuclear structure, mitotic figures, etc., are

by this method well preserved, whereas these are considerably distorted in dried films. The initial stages in the preparation of wet films are the same as above, but instead of being dried in air they are placed, while still wet, film downwards in the fixative. The following are some of the best fixing methods:—

(a) A saturated solution of perchloride of mercury in 75 per cent sodium chloride; fix for five minutes. Then place the films for half an hour, with occasional gentle shaking, in 75 per cent sodium chloride solution to wash out the corrosive sublimate; they are thereafter washed in successive strengths of methylated spirit. After this treatment the films are stained and treated as if they were sections.

(b) Formol-alcohol—formalin 1 part, absolute alcohol 9. Fix films for three minutes; then wash well in methylated spirit. This is an excellent and very rapid method.

(c) Another excellent method of fixing has been devised by Gulland. The fixing solution has the composition—absolute alcohol 25 c.c., pure ether 25 c.c., alcoholic solution of corrosive sublimate (2 gm. in 10 c.c. of alcohol) about 5 drops. The films are placed in this solution for five minutes or longer. They are then washed well in water, and are ready for staining. A contrast stain can be applied at the same time as the fixing solution, by saturating the 25 c.c. of alcohol with eosin before mixing. Thereafter the bacteria, etc., may be stained with methylene-blue or other stain, as described below. This method has the advantage over (a) that, as a small amount of corrosive sublimate is used, less washing is necessary to remove it from the preparation, and deposits are less liable to occur.

**3. Examination of Bacteria in Tissues.**—For the examination of bacteria in the tissues, the latter must be fixed and hardened, in preparation for being cut with a microtome. Fixation consists in so treating a tissue that it shall permanently maintain, as far as possible, the condition it was in when removed from the body. Hardening consists in giving such a fixed tissue sufficient consistence to enable a thin section of it to be cut. A tissue, after being hardened, may be cut in a freezing microtome (*e.g.* Cathcart's or one of the newer instruments in which the freezing is accomplished by compressed carbonic acid gas), but far finer results can be obtained by embedding the tissue in solid paraffin and cutting with some of the more delicate microtomes of which, for pathological purposes, the small Cambridge rocker is by far the best. For bacteriological purposes embedding in celloidin is not advisable, as the celloidin takes on the aniline dyes which are used for staining bacteria, and is apt thus to spoil the preparation, and besides thinner sections can be obtained by the paraffin method.

**The Fixation and Hardening of Tissues.**—The following are amongst the best methods for bacteriological purposes:—

(a) *Absolute alcohol* may be used for the double purpose of fixing and hardening. If the piece of tissue is not more than  $\frac{1}{8}$  inch in thickness it



is sufficient to keep it in this reagent for a few hours. If the pieces are thicker a longer exposure is necessary, and in such cases it is better to change the alcohol at the end of the first twenty-four hours. The tissue must be tough without being hard, and the necessary consistence, as estimated by feeling with the fingers, can only be judged of after some experience. If the tissues are not to be cut at once, they may be preserved in 50 per cent spirit.

(b) *Formol-alcohol*—formalin 1, absolute alcohol 9. Fix for not more than twenty-four hours; then place in absolute alcohol if the tissue is to be embedded at once, in 50 per cent spirit if it is to be kept for some time. For small pieces of tissue fixation for twelve hours or even less is sufficient. The method is a rapid and very satisfactory one.

(c) *Corrosive sublimate* is an excellent fixing agent. It is best used as a saturated solution in 75 per cent sodium chloride solution. Dissolve the sublimate in the salt solution by heat; the separation of crystals on cooling shows that the solution is saturated. For small pieces of tissue  $\frac{1}{4}$  inch in thickness, twelve hours' immersion is sufficient. If the pieces are larger, twenty-four hours is necessary. They should then be tied up in a piece of gauze, and placed in a stream of running water for from twelve to twenty-four hours, according to the size of the pieces, to wash out the excess of sublimate. They are then placed for twenty-four hours in each of the following strengths of methylated spirit (free from naphtha<sup>1</sup>): 30 per cent, 60 per cent, and 90 per cent. Finally they are placed in absolute alcohol for twenty-four hours and are then ready to be prepared for cutting.

If the tissue is very small, as in the case of minute pieces removed for diagnosis, the stages may be all compressed into twenty-four hours. In fact after fixation in corrosive the tissue may be transferred directly to absolute alcohol, the perchloride of mercury being removed after the sections are cut, as will be afterwards described.

(d) *Methylated Spirit*.—Small pieces of tissue may be placed in methylated spirit, which is to be changed after the first day. In from six to seven days they will be hardened. If the pieces are large, a longer time is necessary.

**The Cutting of Sections.**—1. *By Means of the Freezing Microtome*.—Pieces of tissue hardened by any of the above methods must have all the alcohol removed from them by washing in running water for twenty-four hours. They are then placed for from twelve to twenty-four hours (according to their size) in a thick syrupy solution containing two parts of gum arabic and one part of sugar. They are then cut on a freezing microtome and placed for a few hours in a bowl of water so that the gum and syrup may dissolve out. They are then stained or they may be stored in methylated spirit.

<sup>1</sup> In Britain ordinary commercial methylated spirit has wood naphtha added to it to discourage its being used as a beverage. The naphtha being insoluble in water a milky fluid results from the dilution of the spirit. By law, chemists can only sell 8 ounces of pure spirit at a time. Most pathological laboratories are, however, licensed by the Excise to buy "industrial spirit," which contains only one-nineteenth of wood naphtha.

2. *Embedding and Cutting in Solid Paraffin.*—This method gives by far the finest results, and should always be adopted when practicable. The principle is the impregnation of the tissue with paraffin in the melted state. This paraffin when it solidifies gives support to all the tissue elements. The method involves that, after hardening, the tissue shall be thoroughly dehydrated, and then thoroughly permeated by some solvent of paraffin which will expel the dehydrating fluid and prepare for the entrance of the paraffin. The solvents most in use are chloroform, cedar oil, xylol, and turpentine; of these chloroform and cedar oil are the best, the former being preferred as it permeates the tissue more rapidly. The more gradually the tissues are changed from reagent to reagent in the processes to be gone through, the more successful is the result. A necessity of the process is an oven with hot-water jacket, in which the paraffin can be kept at a constant temperature just above its melting-point, a gas regulator, *e.g.* Reichert's, being of course necessary. The tissues occurring in pathological work have a tendency to become brittle if overheated, and therefore the best results are not obtained by using paraffin melting about 58° C., such as is employed in most biological laboratories. We have used for some years a mixture of one part of paraffin, melting at 48°, and two parts of paraffin melting at 54° C. This mixture has a melting-point between 52° and 53° C., and it serves all ordinary purposes well. An excellent quality of paraffin is that known as the "Cambridge paraffin," but many scientific-instrument makers supply paraffins which, for ordinary purposes, are quite as good, and much cheaper. The successive steps in the process of paraffin embedding are as follows:—

1. Pieces of tissue, however hardened, are placed in fresh absolute alcohol for twenty-four hours in order to their complete dehydration.

2. Transfer now to a mixture of equal parts of absolute alcohol and chloroform for twenty-four hours.

3. Transfer to pure chloroform for twenty-four hours or longer. At the end of this time the tissues should sink or float heavily.

4. Transfer now to a mixture of equal parts of chloroform and paraffin and place on the top of the oven for from twelve to twenty-four hours. If the temperature there is not sufficient to keep the mixture melted then they must be put inside.

5. Place in pure melted paraffin in the oven for twenty-four hours. For holding the paraffin containing the tissues, small tin dishes such as are used by pastry-cooks will be found very suitable. There must be a considerable excess of paraffin over the bulk of tissue present, otherwise sufficient chloroform will be present to vitiate the final result and not give the perfectly hard block obtained with pure paraffin. With experience, the persistence of the slightest trace of chloroform can be recognised by smell.

In the case of very small pieces of tissue the time given for each stage may be much shortened, and where haste is desirable Nos. 2 and 4 may be omitted. Otherwise it is better to carry out the process as described. When it is advisable to avoid all shrinkage it is well to change the paraffin every few hours during the embedding process.

6. Cast the tissues in blocks of paraffin as follows: Pairs of L-shaped pieces of metal made for the purpose by instrument makers must be at hand. By laying two of these together on a glass plate, a rectangular trough is formed. This is filled with melted paraffin taken from a stock in a separate dish. In it is immersed the piece of tissue, which is lifted out of its pure paraffin bath with heated forceps. The direction in which it is to be cut must be noted before the paraffin becomes opaque. When the paraffin has begun to set, the glass plate and trough have cold water run over them. When the block is cold, the metal L's are broken off, and, its edges having been pared, it is stored in a pill-box.

*The Cutting of Paraffin Sections.*—Sections must be cut as thin as possible, the Cambridge rocking microtome being, on the whole, most suitable. They should not exceed  $8\ \mu$  in thick-

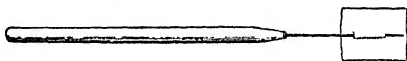


FIG. 43.—Needle with square of paper on end for manipulating paraffin sections.

ness, and ought, if possible, to be about  $4\ \mu$ . For their manipulation it is best to have two needles on handles, two camel's-hair brushes on handles, and a needle with a rectangle of stiff writing paper fixed on it as in the diagram (Fig. 43). When cut, sections are floated on the surface of a beaker of water kept at a temperature about  $10^{\circ}\text{C}$ . below the melting-point of the paraffin. On the surface of the warm water they become perfectly flat.

*Fixation on Ordinary Slides.* (a) *Gulland's Method.*—A supply of slides well cleaned being at hand, one of them is thrust obliquely into the water below the section, a corner of the section is fixed on it with a needle and the slide withdrawn. The surplus of water being wiped off with a cloth, the slide is placed on a support, with the section downwards, and allowed to remain on the top of the paraffin oven or in a bacteriological incubator for from twelve to twenty-four hours. It will then be sufficiently fixed on the slide to withstand all the manipulations necessary during staining and mounting.

(b) *Fixation by Mann's Method.*—This has the advantage of being more rapid than the previous one. A solution of albumin is prepared by mixing the white of a fresh egg with ten parts of distilled water and filtering. Slides are made perfectly clean with alcohol. One is dipped into the solution and its edge is then drawn over one surface of another slide so as to leave on it a thin film of albumin. This is repeated with the others. As each is thus coated, it is leant, with the film down-

wards, on a ledge till dry, and then the slides are stored in a wide stoppered jar till needed. The floating out is performed as before. The albuminised side of the slide is easily recognised by the fact that if it is breathed on, the breath does not condense on it. The great advantage of this method is that the section is fixed after twenty to thirty minutes' drying at 37° C. If the tissue has been hardened in any of the bichromate solutions and embedded in paraffin, this or some corresponding method of fixing the sections on the slide must be used.

*Preparation of Paraffin Sections for Staining.*—Before staining, the paraffin must be removed from the section. This is best done by dropping on xylol out of a drop bottle. When the paraffin is dissolved out, the superfluous xylol is wiped off with a cloth and a little absolute alcohol dropped on. When the xylol is removed the superfluous alcohol is wiped off and a little 50 per cent methylated spirit dropped on. During these procedures sections must on no account be allowed to dry. The sections are now ready to be stained. Deposits of crystals of corrosive sublimate often occur in sections which have been fixed by this reagent. These can be removed by placing the sections, before staining, for a few minutes in equal parts of Gram's iodine solution (p. 99) and water, and then washing out the iodine with methylated spirit.

To save repetition we shall in treating of stains suppose that, with paraffin sections, the above preliminary steps have already been taken, and further that sections cut by a freezing microtome are also in spirit and water.

**Dehydration and Clearing.**—It is convenient, first of all, to indicate the final steps to be taken after a specimen is stained. *Dry films* after being stained are washed in water, dried and mounted in xylol balsam; *wet films* and *sections* must be dehydrated, cleared, and then mounted in xylol balsam.

*Dehydration* is most commonly effected with absolute alcohol. Alcohol, however, sometimes decolorises the stained organisms more than is desirable, and therefore Weigert devised the following method of dehydrating and clearing by aniline oil, which, though it may decolorise somewhat, does not do so to the same extent as alcohol. As much as possible of the water being removed, the section placed on a slide is partially dried by draining with fine blotting-paper. Some aniline oil is placed on the section and the slide moved to and fro. The section is dehydrated and becomes clear. The process may be accelerated by heating gently. The preparation is then treated with a mixture of two parts of aniline oil and one part of xylol, and then with xylol alone, after which it is mounted in xylol balsam. Balsam as ordinarily supplied has often an acid reaction, and

preparations stained with aniline dyes are apt to fade when mounted in it. It is accordingly a great advantage to use the acid-free balsam supplied by Grüber. Paraffin sections can usually be dehydrated and cleared by the mixture of aniline oil and xylol alone.

Sections stained for bacteria should always be *cleared*, at least finally, in xylol, for the same reason that xylol balsam is to be used for mounting films, viz. that it dissolves out aniline dyes less readily than such clearing reagents as clove oil, etc. Xylol, however, requires the previous dehydration to have been more complete than clove oil, which will clear a section readily when the dehydration has been only partially effected by, say, methylated spirit. If a little decolorisation of a section is still required before mounting, clove oil may be used to commence the clearing, the process being finished with xylol. With a little experience the progress, not only of these processes but also of staining, can be very accurately judged of by observing the appearances under a low objective.

### THE STAINING OF BACTERIA.

**Staining Principles.**—To speak generally, the protoplasm of bacteria reacts to stains in a manner similar to the nuclear chromatin, though sometimes more and sometimes less actively. The bacterial stains *par excellence* are the basic aniline dyes. These dyes are more or less complicated compounds derived from the coal-tar product aniline ( $C_6H_5 \cdot NH_2$ ). Many of them have the constitution of salts. Such compounds are divided into two groups according as the staining action depends on the basic or the acid portion of the molecule. Thus the acetate of rosaniline derives its staining action from the rosaniline. It is therefore called a basic aniline dye. On the other hand, ammonium picrate owes its action to the picric acid part of the molecule. It is therefore termed an acid aniline dye. These two groups have affinities for different parts of the animal cell. The basic stains have a special affinity for the nuclear chromatin, the acid for the protoplasm and various formed elements. Thus it is that the former—the basic aniline dyes—are especially the bacterial stains.

The number of basic aniline stains is very large. The following are the most commonly used :—

*Violet Stains.*—Methyl-violet, R-5R (synonyms : Hoffmann's violet, dahlia).

Gentian-violet (synonyms : benzyl-violet, Pyoktanin).

Crystal violet.

*Blue Stains*.—Methylene-blue<sup>1</sup> (synonym : phenylene-blue).

Victoria-blue.

Thionin-blue.

*Red Stains*.—Basic fuchsin (synonyms : basic rubin, magenta).

Safranin (synonyms : fuchsia, Giroflé).

*Brown Stain*.—Bismarck - brown (synonyms : vesuvin, phenylene-brown).

It is of the greatest importance that the stains used by the bacteriologist should be good, and therefore it is advisable to obtain those prepared by Grüber of Leipzig. One is then perfectly sure that one has got the right stain.

Of the stains specified, the violets and reds are the most intense in action, especially the former. It is thus easy in using them to overstain a specimen. Of the blues, methylene-blue probably gives the best differentiation of structure, and it is difficult to overstain with it. Thionin-blue also gives good differentiation and does not readily overstain. Its tone is deeper than that of methylene-blue and it approaches the violets in tint. Bismarck-brown is a weak stain, but is useful for some purposes. Formerly it was much used in photomicrographic work, as it was less actinic than the other stains. It is not, however, needed now, on account of the improved sensitiveness of plates.

It is most convenient to keep saturated alcoholic solutions of the stains made up, and for use to filter a little into about ten times its bulk of distilled water in a watch-glass. A solution of good body is thus obtained. Most bacteria (except those of tubercle, leprosy, and a few others) will stain in a short time in such a fluid. Watery solutions may also be made up, *e.g.* a saturated watery solution of methylene-blue or a 1 per cent solution of gentian-violet. Stains must always be filtered before use; otherwise there may be deposited on the preparation granules which it is impossible to wash off. The violet stains in solution in water have a great tendency to decompose. Only small quantities should therefore be prepared at a time.

*The Staining of Cover-glass Films*.—Films are made from cultures as described above. The cover-glass may be floated on the surface of the stain in a watch-glass, or the cover-glass held in forceps with film side uppermost may have as much stain poured on it as it will hold. When the preparation has been exposed for the requisite time, usually a few minutes, it is well washed in tap water in a bowl, or with distilled water with such a simple contrivance as that figured (Fig. 44). The figure explains itself.

<sup>1</sup> This is to be distinguished from methyl-blue, which is a different compound.

When the film has been washed the surplus of water is drawn off with a piece of filter-paper, the preparation is carefully dried high over a flame, a drop of xylol balsam is applied, and the cover-glass mounted on a slide. It is sometimes advantageous to examine films in a drop of water in place of balsam. The films can be subsequently dried and mounted permanently. In the case of tubercle, special stains are necessary (p. 100), but with this exception, practically all bacterial films made from cultures can be stained in this way. Some bacteria, *e.g.* typhoid, glanders, take up the stains rather slowly, and for these the more intensive stains, red or violet, are to be preferred.

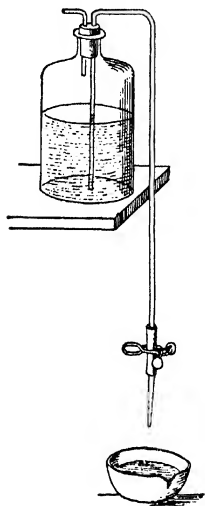


FIG. 44.—Syphon wash-bottle for distilled water used in washing preparations.

Films of *fluids from the body* (blood, pus, etc.) can be generally stained in the same way, and this is often quite sufficient for diagnostic purposes. The blue dyes are here preferable, as they do not readily overstain. In the case of such fluids, if the histological elements also claim attention it is best first to stain the cellular protoplasm with a one to two per cent watery solution of eosin (which is an acid dye), and then to use a blue which will stain the bacteria and the nuclei of the cells. The Romanowsky stains (*v. p.* 105) are here most useful, as by these the preparations are fixed as well as stained. Fixation by heat which is apt to injure delicate cellular structures is thus avoided. In the case of films made from urine, where there is little or no albuminous matter present, the bacteria may be imperfectly fixed on

the slide, and are thus apt to be washed off. In such a case it is well to modify the staining method. A drop of stain is placed on a slide, and the cover-glass, film-side down, lowered upon it. After the lapse of the time necessary for staining, a drop of water is placed at one side of the cover-glass and a little piece of filter-paper at the other side. The result is that the stain is sucked out by the filter-paper. By adding fresh drops of water and using fresh pieces of filter-paper, the specimen is washed without any violent application of water, and the bacteria are not displaced.

For the general staining of films a saturated watery solution of methylene-blue will be found to be the best stain to commence with, the Gram method (*v. infra*) is then applied, and subsequently any special stains which may appear advisable.

**The Use of Mordants and Decolorising Agents.**—In films of blood and pus, and still more so in sections of tissues, if the above methods are used, the tissue elements may be stained to such an extent as to quite obscure the bacteria. Hence many methods have been devised in which the general principle may be said to be (*a*) the use of substances which, while increasing the staining power, tend to fix the stain in the bacteria, and (*b*) the subsequent treatment by substances which decolorise the overstained tissues to a greater or less extent, while they leave the bacteria coloured. The staining capacity of a solution may be increased—

(*a*) By the addition of substances such as carbolic acid, aniline oil, or metallic salts.

(*b*) By the addition of alkalies, such as caustic potash or ammonium carbonate, in weak solution.

(*c*) By the employment of heat.

(*d*) By long duration of the staining process.

As decolorising agents we use chiefly mineral acids (hydrochloric, nitric, sulphuric), vegetable acids (especially acetic acid), alcohol (either methylated spirit or absolute alcohol), or a combination of spirit and acid, *e.g.* methylated spirit with a drop or two of hydrochloric acid added, also various oils, *e.g.* aniline, clove, etc. In most cases about thirty drops of acetic acid in a bowl of water will be sufficient to remove the excess of stain from over-stained films and sections. More of the acid may, of course, be added if necessary.

Hot water also decolorises to a certain extent; over-stained films can be readily decolorised by placing a drop of water on the film and heating gently over a flame.

When preparations have been sufficiently decolorised by an acid, they should be well washed in tap water, or in distilled water with a little lithium carbonate added.

The methods embracing the use of a stain with a mordant, and a decoloriser, are very numerous, and we can only enumerate the best of them.

Different organisms take up and retain the stains with various degrees of intensity, and thus duration of staining and decolorising must be modified accordingly. We sometimes have to deal with bacteria which show a special tendency to be decolorised. This tendency can be obviated by adding a little



of the stain to the alcohol, or aniline oil, employed in dehydration. In the latter case a little of the stain is rubbed down in the oil. The mixture is allowed to stand. After a little time a clear layer forms on the top with stain in solution, and this can be drawn off with a pipette.

When methylene-blue, methyl-violet, or gentian-violet is used, the stain can, after the proper degree of decolorisation has been reached, be fixed in the tissues by treating for a minute with ammonium molybdate ( $2\frac{1}{2}$  per cent in water).

**The Formulæ of some of the more commonly used Stain Combinations.**

1. *Löffler's Methylene-blue.*

|   |         |
|---|---------|
| Saturated solution of methylene-blue in alcohol             | 30 c.c. |
| Solution of potassium hydrate in distilled water (1-10,000) | 100 „   |

(This dilute solution may be conveniently made by adding 1 c.c. of a 1 per cent solution to 99 c.c. of water.)

*Sections* may be stained in this mixture for from a quarter of an hour to several hours. They do not readily overstain. The tissue containing the bacteria is then decolorised if necessary with  $\frac{1}{2}$ -1 per cent acetic acid, till it is a pale blue-green. The section is washed in water, rapidly dehydrated with alcohol or aniline oil, cleared in xylol, and mounted.

The tissue may be contrast stained with eosin. If this is desired, after decolorisation wash with water, place for a few seconds in 1 per cent solution of eosin in absolute alcohol, rapidly complete dehydration with pure absolute alcohol, and proceed as before.

*Films* may be stained with Löffler's blue by five minutes' exposure or longer in the cold. They usually do not require decolorisation, as the tissue elements are not overstained.

2. *Kühne's Methylene-blue.*

|                               |         |
|-------------------------------|---------|
| Methylene-blue                | 1.5 gr. |
| Absolute alcohol              | 10 c.c. |
| Carbolic acid solution (1-20) | 100 „   |

Stain and decolorise as with Löffler's blue, or decolorise with very weak hydrochloric acid (a few drops in a bowl of water).

3. *Carbol-Thionin-blue.*—Make up a stock solution consisting of 1 gramme of thionin-blue dissolved in 100 c.c. carbolic acid solution (1-40). For use, dilute 1 volume with 3 of water and filter. Stain sections for five minutes or upwards. Wash very thoroughly with water, otherwise a deposit of crystals may occur in the subsequent stages. Decolorise with very weak acetic acid. A few drops of the acid added to a bowl of water are quite sufficient. Wash again thoroughly with water. Dehydrate with absolute alcohol. Thionin-blue stains more deeply than methylene-blue, and gives equally good differentiation. It is very suitable for staining typhoid and glanders bacilli in sections. Cover-glass preparations stained by this method do not usually require decolorisation. As a contrast stain, 1 per cent watery solution of eosin may be used before staining with the thionin.

4. *Gentian-violet in Aniline Oil Water.*—Two solutions have here to be made up. (a) Aniline oil water. Add about 5 c.c. aniline oil to 100 c.c. distilled water in a flask, and shake violently till as much as possible of the oil has dissolved. Filter and keep in a covered bottle

to prevent access of light. (b) Make a saturated solution of gentian-violet in alcohol. When the stain is to be used, 1 part of (b) is added to 10 parts of (a), and the mixture filtered. The mixture should be made not more than twenty-four hours before use. Stain sections for a few minutes; then decolorise with methylated spirit. Sometimes it is advantageous to add to the methylated spirit a little hydrochloric acid (2-3 minims to 100 c.c.). This staining solution is not so much used by itself, as in Gram's method, which is presently to be described.

5. *Carbol-Gentian-Violet*.—1 part of saturated alcoholic solution of gentian-violet is mixed with 10 parts of 5 per cent solution of carbolic acid. It is used as No. 4.

6. *Carbol-Fuchsin* (see p. 101).—This is a very powerful stain, and, when used in the undiluted condition,  $\frac{1}{2}$ -1 minute's staining is usually sufficient. It is better, however, to dilute with from five to ten times its volume of water and stain for a few minutes. In this form it has a very wide application. Methylated spirit with or without a few drops of acetic acid is the most convenient decolorising agent. Then dehydrate thoroughly, clear, and mount.

**Gram's Method and its Modifications.**—In the methods already described the tissues, and more especially the nuclei, retain some stain when decolorisation has reached the point to which it can safely go without the bacteria themselves being affected. In the method of Gram, now to be detailed, this does not occur, for the stain can here be removed completely from the ordinary tissues, and left only in the bacteria. All kinds of bacteria, however, do not retain the stain in this method, and therefore in the systematic description of any species it is customary to state whether it is, or is not, stained by Gram's method—by this is meant, as will be understood from what has been said, *whether the particular organism retains the colour after the latter has been completely removed from the tissues*. It must, however, be remarked that some tissue elements may retain the stain as firmly as any bacteria, *e.g.* keratinised epithelium, calcified particles, the granules of mast cells, and sometimes altered red blood corpuscles, etc.

In Gram's method the essential feature is the treating of the tissue, after staining, with a solution of iodine. This solution is spoken of as Gram's solution, and has the following composition:—

|                            |         |
|----------------------------|---------|
| Iodine . . . . .           | 1 part  |
| Potassium iodide . . . . . | 2 parts |
| Distilled water . . . . .  | 300 „   |

The following is the method:—

1. Stain in aniline oil gentian-violet or in carbol-gentian-violet (*vide supra*) for about five minutes, and wash in water.
2. Treat the section or film with Gram's solution till its colour becomes a purplish black—generally about half a minute or a minute is sufficient for the action to take place.

3. Decolorise with absolute alcohol or methylated spirit till the colour has almost entirely disappeared, the tissues having only a faint violet tint.

4. Dehydrate completely, clear with xylol and mount. In the case of film preparations, the specimen is simply washed in water, dried and mounted.

In stage (3) the process of decolorisation is more satisfactorily performed by using clove oil after sufficient dehydration with alcohol, the clove oil being afterwards removed by xylol.

As a *contrast* stain for the tissues carmalum or lithia carmine is used before staining with gentian-violet (1). As a contrast stain for other bacteria which are decolorised by Gram's method carbol-fuchsin diluted with ten volumes of water or a saturated watery solution of Bismarck-brown may be used before stage (4).

The following modifications of Gram's method may be given:—

1. *Wiegert's Modification*.—The contrast staining of the tissues and stages (1) and (2) are performed as above.

(3) After using the iodine solution the preparation is dried by blotting and then decolorised by aniline-xylol (aniline-oil 2, xylol 1).

(4) Wash well in xylol and mount in xylol balsam. Film preparations after being washed in xylol may be dried and thereafter dilute carbol-fuchsin may be used to stain bacteria which have been decolorised.

This modification probably gives the most uniformly successful results.

2. *Nicoll's Modification*.—Carbol-gentian-violet is used as the stain. Treatment with iodine is carried out as above and decolorisation is effected with a mixture of acetone (1 part) and alcohol (2 parts).

3. *Kühne's Modification*.—(1) Stain for five minutes in a solution made up of equal parts of saturated alcoholic solution of crystal-violet ('KrySTALL-violet') and 1 per cent solution of ammonium carbonate.

(2) Wash in water.

(3) Place for two to three minutes in Gram's iodine solution, or in the following modification by Kühne:—

|                            |         |
|----------------------------|---------|
| Iodine . . . . .           | 2 parts |
| Potassium iodide . . . . . | 4 „     |
| Distilled water . . . . .  | 100 „   |

For use, dilute with water to make a sherry-coloured solution.

(4) Wash in water.

(5) Decolorise in a saturated alcoholic solution of fluorescein (a saturated solution in methylated spirit does equally well).

(6) Dehydrate, clear and mount.

There is great variability in the avidity with which organisms stained by Gram retain the dye when washed with alcohol, and sometimes difficulty is experienced in saying whether an organism does or does not stain by this method.

**Stain for Tubercle and other Acid-fast Bacilli.**—These bacilli cannot be well stained with a simple watery solution of a basic aniline dye. This fact can easily be tested by attempting to stain a film of a tubercle culture with such a solution. They require a powerful stain containing a mordant, and must be exposed to the stain for a long time, or its action may be aided

by a short application of heat. When once stained, however, they resist decolorising even with very powerful acids; they are therefore called "acid-fast." The smegma bacillus also resists decolorising with strong acids (p. 254), and a number of other acid-fast bacilli have recently been discovered (p. 252). Any combination of gentian-violet or fuchsin with aniline oil or carbolic acid or other mordant will stain the bacilli named, but the following methods are most commonly used:—

*Ziehl-Neelsen Carbol-Fuchsin Stain.*

|                                    |           |          |
|------------------------------------|-----------|----------|
| Basic fuchsin                      | . . . . . | 1 part   |
| Absolute alcohol                   | . . . . . | 10 parts |
| Solution of carbolic acid (1 : 20) | . . . . . | 100 „    |

1. Place the specimen in this fluid, and having heated it till steam rises, allow it to remain there for five minutes, or allow it to remain in the cold stain for from twelve to twenty-four hours. (Films and paraffin sections are usually stained with hot stain, loose sections with cold; in hot stain the latter shrink.)

2. Decolorise with 20 per cent solution of strong sulphuric acid, nitric acid, or hydrochloric acid, in water. In this the tissues become yellow.

3. Wash well with water. The tissues will regain a faint pink tint. If the colour is distinctly red, the decolorisation is insufficient, and the specimen must be returned to the acid. As a matter of practice, it is best to remove the preparation from the acid every few seconds and wash in water, replacing the specimen in the acid and re-washing till the proper pale pink tint is obtained. Then wash in alcohol for half a minute and replace in water.

4. Contrast stain with a saturated watery solution of methylene-blue for half a minute, or with saturated watery Bismarck-brown for from two to three minutes.

5. Wash well with water. In the case of films, dry and mount. In the case of sections, dehydrate, clear and mount.

*Fraenkel's Modification of the Ziehl-Neelsen Stain.*

Here the process is shortened by using a mixture containing both the decolorising agent and the contrast stain.

The sections or films are stained with the carbol-fuchsin as above described, and then placed in the following solution:—

|   |           |          |
|---|-----------|----------|
| Distilled water                           | . . . . . | 50 parts |
| Absolute alcohol                          | . . . . . | 30 „     |
| Nitric acid                               | . . . . . | 20 „     |
| Methylene-blue in crystals to saturation. |           |          |

They are treated with this till the red colour has quite disappeared and been replaced by blue. The subsequent stages are the same as in No. 5, *supra*.

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Leprosy bacilli are stained in the same way, but are rather more easily decolorised than tubercle bacilli, and it is better to use only 5 per cent sulphuric acid in decolorising.

In the case of specimens stained either by the original Ziehl-Neelsen method, or by Fraenkel's modification, the tubercle or leprosy bacilli ought to be bright red, and the tissue blue or brown, according to the contrast stain used. Other bacteria which may be present are also coloured with the contrast stain.

**The Staining of Spores.**—If bacilli containing spores are stained with a watery solution of a basic aniline dye the spores remain unstained. The spores either take up the stain less readily than the protoplasm of the bacilli or they have a resisting envelope which prevents the stain penetrating to the protoplasm. Like the tubercle bacilli, when once stained they retain the colour with considerable tenacity. The following is the simplest method for staining spores:—

1. Stain cover-glass films as for tubercle bacilli.
2. Decolorise with 1 per cent sulphuric acid in water or with methylated spirit. This removes the stain from the bacilli.
3. Wash in water.
4. Stain with saturated watery methylene-blue for half a minute.
5. Wash in water, dry, and mount in balsam.

The result is that the spores are stained red, the protoplasm of the bacilli blue.

The spores of some organisms lose the stain more readily than those of others, and for some, methylated spirit is a sufficiently strong decolorising agent for use. If sulphuric acid stronger than 1 per cent is used the spores of many bacilli are readily decolorised.

**Möller's Method.**—The following method, recommended by Möller, is much more satisfactory than the previous. Before being stained, the films are placed in chloroform for 2 minutes, and then in a 5 per cent solution of chromic acid for  $\frac{1}{2}$ -2 minutes, the preparation being well washed after each reagent. Thereafter they are stained and decolorised as above.

**The Staining of Capsules.**—The two following methods may be recommended in the case of capsulated bacteria:—

(a) *Welch's Method.*—This depends on the fact that in many cases the capsules can be fixed with glacial acetic acid.

Films when still wet are placed in this acid for a few seconds.

The superfluous acid is removed with filter-paper and the preparation is treated with gentian-violet in aniline oil water repeatedly till all the acetic acid is removed.

Then wash with 1-2 per cent solution of sodium chloride and examine in the same solution.

The capsule appears as a pale violet halo around the deeply stained bacterium.

(b) *Richard Muir's Method* (as recently modified).

1. The film containing the bacteria must be very thin. It is dried and stained in filtered carbol-fuchsin for half a minute, the preparation being gently heated.

2. Wash slightly with spirit and then well in water.

3. Place in following mordant for a few seconds :—

|   |         |
|---|---------|
| Saturated solution of corrosive sublimate . . . | 2 parts |
| Tannic acid solution—20 per cent . . .          | 2 „     |
| Saturated solution of potash alum . . .         | 5 „     |

4. Wash well in water.

5. Treat with methylated spirit for about a minute.

The preparation has a pale reddish appearance.

6. Wash well in water.

7. Counterstain with watery solution of ordinary methylene-blue for half a minute.

8. Dehydrate in alcohol, clear in xylol, and mount in balsam.

The bacteria are a deep crimson and the capsules of a blue tint. The capsules of bacteria in cultures may sometimes be demonstrated by this method.

**The Staining of Flagella.**—The staining of the flagella of bacteria is the most difficult of all bacteriological procedures, and it requires considerable practice to ensure that good results shall be obtained. Many methods have been introduced, of which the two following are the most satisfactory.

*Preparation of Films.*—In all the methods of staining flagella, young cultures on agar should be used, say a culture incubated for from ten to eighteen hours at 37° C. A very small portion of the growth is taken on the point of a platinum needle and carefully mixed in a little water in a watch-glass; the amount should be such as to produce scarcely any turbidity in the water. A film is then made by placing a drop on a clean cover-glass and carefully spreading it out with the needle. It is allowed to dry in the air and is then passed twice or thrice through a flame, care being taken not to over-heat it. The cover-glasses used should always be cleaned in the mixture of sulphuric acid and potassium bichromate described on page 87.

#### 1. *Pittfield's Method as modified by Richard Muir.*

Prepare the following solutions :—

##### A. *The Mordant.*

|  |         |
|--|---------|
| Tannic acid, 10 per cent watery solution, filtered . . . | 10 c.c. |
| Corrosive sublimate, saturated watery solution . . .     | 5 „     |
| Alum, saturated watery solution . . .                    | 5 „     |
| Carbol-fuchsin ( <i>vide</i> p. 101) . . .               | 5 „     |

Mix thoroughly. A precipitate forms, which must be allowed to deposit, either by centrifugalising or simply by allowing to stand. Remove the clear fluid with a pipette and transfer to a clean bottle. The mordant keeps well for one or two weeks.

##### B. *The Stain.*

|  |         |
|--|---------|
| Alum, saturated watery solution . . .              | 10 c.c. |
| Gentian-violet, saturated alcoholic solution . . . | 2 „     |

The stain should not be more than two or three days old when used. It may be substituted in the mordant in place of the carbol-fuchsin.

The film having been prepared as above described, pour over it as much of the mordant as the cover-glass will hold. Heat gently over a flame till steam begins to rise, allow to steam for about a minute, and then wash well in a stream of running water for about two minutes. Then dry carefully over the flame, and when thoroughly dry pour on some of the stain. Heat as before, allowing to steam for about a minute, wash well in water, dry and mount in a drop of xylol balsam.

This method has yielded the best results in our hands.

## 2. *Van Ermengem's Method for Staining Flagella.*

The films are prepared as above described. Three solutions are here necessary :—

Solution A. (*Bain fixateur*)—

|                                 |           |         |
|---------------------------------|-----------|---------|
| Osmic acid, 2 per cent solution | . . . . . | 1 part  |
| Tannin, 10-25 per cent solution | . . . . . | 2 parts |

Place the films in this for one hour at room temperature, or heat over a flame till steam rises and keep in the hot stain for five minutes. Wash with distilled water, then with absolute alcohol for three to four minutes, and again in distilled water, and treat with

Solution B. (*Bain sensibilisateur*)—

.5 per cent solution of nitrate of silver in distilled water. Allow films to be in this a few seconds. Then without washing transfer to

Solution C. (*Bain reducteur et renforçateur*)—

|                         |           |          |
|-------------------------|-----------|----------|
| Gallic acid             | . . . . . | 5 grm.   |
| Tannin                  | . . . . . | 3 „      |
| Fused potassium acetate | . . . . . | 10 „     |
| Distilled water         | . . . . . | 350 c.c. |

Keep in this for a few seconds. Then treat again with Solution B till the preparation begins to turn black. Wash, dry, and mount.

It is better, as Mervyn Gordon recommends, to leave the specimen in B for two minutes and then to transfer to C for one and a half to two minutes, and not to transfer again to B. It will also be found an advantage to use a fresh supply of C for each preparation, a small quantity being sufficient. The beginner will find the typhoid bacillus or the bacillus coli communis very suitable organisms to stain by this method.

Although the results obtained by this method are sometimes excellent, they vary considerably. Frequently both the organisms and flagella appear of abnormal thickness. This is due to the fact that the process on which the method depends is a precipitation rather than a true staining. The pictures on the whole are less faithful than in the first method.

**Staining of Spirochæte in Sections.**—The following impregnation method, which is practically that of Ramon-y-Cajal

for nerve fibrillae, has been applied for this purpose by Levaditi and gives excellent results.

(1) The tissues, which ought to be in thin slices, about 1 mm. in thickness, are best fixed in 10 per cent formalin solution for twenty-four hours.

(2) They are washed for an hour in water and then brought into 96 per cent alcohol for twenty-four hours.

(3) They are then placed in 1.5 per cent solution of nitrate of silver in a dark bottle, and are kept in an incubator at 37° C. for three days.

(4) They are washed in water for about twenty minutes, and are thereafter placed in the following mixture, namely :—

Pyrogallic acid, 4 parts.

Formalin, 5 parts.

Distilled water up to 100 parts.

They are kept in this mixture in a dark bottle for forty-eight hours at room temperature.

(5) They are then washed in water for a few minutes, taken through increasing strengths of alcohol, and embedded in paraffin in the usual way. The sections ought to be as thin as possible. In satisfactory preparations the spirochaetes appear of an almost black colour against the pale yellow background of the tissues. The latter can be contrast stained by weak carbol-fuchsin or by toluidin blue.

(For the staining of spirochaetes in films see p. 107.)

**The Romanowsky Stains.**—Within recent years the numerous modifications of the Romanowsky stain have been extensively used. The dye concerned is the compound which is formed when watery solutions of medicinal methylene-blue and water-soluble eosin are brought together. This compound is insoluble in water but soluble in alcohol—the alcohol employed being methyl alcohol. The stain was originally used by Romanowsky for the malarial parasite, and its special quality is that it imparts to certain elements, such as the chromatin of this organism, a reddish-purple hue. This was at first thought to be simply due to the combination of the methylene-blue and the eosin, but it is now recognised that certain changes, such as occur in methylene-blue solutions with age, are necessary. In the modern formulæ these changes are brought about by treatment with alkalis, especially alkaline carbonates, as was first practised by Unna in the preparation of his polychrome methylene-blue. It is not certainly known to what particular



new body the reddish hue is due, but it may be to methyl-violet or to methyl-azure, both of which result from the action of alkali on methylene-blue. The stains are much used in staining blood-films (in which the characters of both nucleus and cytoplasm are beautifully brought out), in staining bacteria in tissues or exudates, the malaria parasite, trypanosomes, the pathogenic spirochaetes (such as the spirochaete pallida), and protozoa generally.

The following are the chief formulæ in use:—

1. *Jenner's Stain*.—This is an excellent blood stain, but is not so good for the study of parasites as the others to be mentioned. In its preparation no alkali is used. It is made by mixing equal parts of (a) a 1·2 to 1·25 per cent solution of Grüber's water soluble eosin (yellow shade) in distilled water and (b) 1 per cent Grüber's medicinal methylene-blue (also a watery solution). The mixture is allowed to stand twenty-four hours, is filtered, and the residue is dried at 55° C.; the powder is shaken up in distilled water, filtered, washed with distilled water and dried. Of the powder, 5 grms. are dissolved in 100 c.c. Merck's methyl alcohol. For use a few drops are placed on the dried unfixed film for one to three minutes, the dye is poured off, and the preparation washed with distilled water till it presents a pink colour; it is then dried between filter-paper and mounted in xylol balsam.

2. *Leishman's Stain*.—The following solutions are prepared: (a) to a 1 per cent solution of medicinal methylene-blue is added 5 per cent sodium carbonate; the mixture is kept at 65° C. for twelve hours and then for ten days at room temperature (25 per cent formalin may be added as a preservative); (b) 1-1000 solution of eosin, extra B.A., in distilled water. Equal volumes of the two solutions are mixed and allowed to stand for six to twelve hours with occasional stirring, the precipitate is collected, filtered, washed with distilled water and dried. For use 15 per cent is dissolved in Merck's methyl alcohol ("for analysis, acetone free") as follows: the powder is placed in a clean mortar, a little of the alcohol is added and well rubbed up with a pestle; the undissolved powder is allowed to settle and the fluid decanted into a dry bottle; the process is repeated with fresh fractions of the solvent till practically all the stain is dissolved, and the bottle is well stoppered; the stain will keep for a long period. For the staining of films a few drops of the stain are placed on the unfixed preparation for fifteen to thirty seconds so as to cover it with a shallow layer (the stain may be conveniently spread over the film with a glass rod) and the film is tilted to and fro so as to prevent drying. This treatment efficiently fixes the film by the action of the methyl alcohol. About double the quantity of distilled water is now dropped on the film, and the stain and diluent are quickly mixed with the rod. Five minutes are now allowed for staining, and the stain is then gently washed off with distilled water. A little of the water is kept on the film for half a minute to intensify the colour contrasts in the various cells. For certain special structures such as Schüffner's dots or Maurer's dots in the malarial parasite a longer staining (up to one hour) may be necessary, and in any case it is well to practise being able to control the depth of the staining effect by observation with a low power objective. If a preparation is to be stained for a long time it

must be kept covered, and if in such cases a granular deposit is formed this may be got rid of by a quick wash with absolute alcohol. If in blood films the red corpuscles appear bluish instead of pink the colour may be restored by washing the film with acetic acid, 1-1500. The film is dried between filter-paper and mounted.

For staining sections a little modification is necessary. A paraffin section is taken into distilled water as usual, the excess of water is drained off, and a mixture of one part of stain and two parts of distilled water is placed on it. The stain is allowed to act for five to ten minutes till the tissue appears a deep Oxford blue; it is then decolorised with 1-1500 acetic acid—the effect being watched under a low-power lens. The blue begins to come out, and the process is allowed to go on till only the nuclei remain blue. The section is then washed with distilled water, rapidly dehydrated with alcohol, cleared and mounted. If, as sometimes happens, the eosin tint be too well marked it can be lightened by the action of 1-7000 solution of caustic soda, this being washed off whenever the desired colour has been attained.

3. *J. H. Wright's Stain*.—In this modification 1 per cent methylene-blue (BX or Ehrlich's rectified) and  $\frac{1}{2}$  per cent sodium carbonate (both in water) are mixed and placed in a Koch's steriliser for an hour. When the fluid is cold, 1-1000 solution of extra B.A. eosin is added till the mixture becomes purplish and a finely granular black precipitate appears in suspension (about 50 c.c. eosin to 100 c.c. methylene-blue solution are required); the precipitate is filtered off and dried without being washed. A saturated solution of this is made in the pure methyl alcohol; this is filtered and diluted by adding to 80 c.c. of the saturated solution 20 c.c. of methyl alcohol. The application of the stain is almost the same as with Leishman's. A few drops are placed on the preparation for a minute for fixation; water is then dropped on till a green iridescent scum appears on the top of the fluid and staining goes on for about two minutes, the stain is then washed off with distilled water and a little is allowed to remain on the film till differentiation is complete; the preparation is carefully dried with filter-paper and mounted.

4. *Giemsa's Stain*.—Giemsa believes that the reddish-blue hue characteristic of the Romanowsky stain is due to the formation of methyl-azure, and he has prepared this by a method of his own under the name "Azur I." From this, by the addition of equal parts of medicinal methylene-blue, he prepares what he calls "Azur II.," and from this again by the addition of eosin he prepares "Azur II.-eosin." The latest formula for the finished stain is as follows:—Azur II.-eosin 3 gr., Azur II. 8 gr., Glycerin (Merck, chemically pure) 250 gr., Methyl alcohol (Kahlbaum, I.) 250 gr. This stain has been extensively used for demonstrating the *Spirochaete pallida*, but it can be used for any other purpose to which the Romanowsky stains are applicable. For the *spirochaete* the following are Giemsa's directions:—

(1) Fix films in absolute alcohol for fifteen to twenty minutes, dry with filter-paper. (2) Dilute stain with distilled water—one drop of stain to 1 c.c. water (the mixture being well shaken). (Sometimes the water is made alkaline by the addition of one drop of 1 per cent potassium carbonate to 10 c.c. water). (3) Stain for fifteen minutes. (4) Wash in brisk stream of distilled water. (5) Drain with filter-paper, dry, and mount in Canada balsam.

With regard to the Jenner and Giemsa stains it is best to obtain the solutions from Grübler ready for use; the powder for Leishman's stain may be obtained from the same source and the solution made up by

condition into contact with the bacteria. The stages of procedure are the following:—

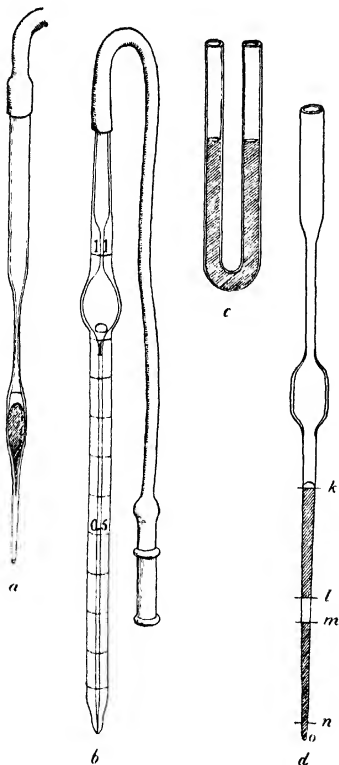
1. Blood is conveniently obtained by pricking the lobe of the ear, which should previously have been washed with a mixture of alcohol and ether and allowed to dry. The blood is drawn up into a Wright's blood capsule (Fig. 47) or into the bulbous portion of a capillary pipette, such as in Fig. 46, *a*. (These pipettes can be readily made by drawing out quill glass tubing in a flame. It is convenient always to have several ready for use.) The pipette is kept in the upright position, one end being closed. For purposes of transit, break off the bulb at the constriction and seal the ends. After the serum has separated from the coagulum the bulb is broken through near its upper end and the serum removed by means of another capillary pipette. The serum is then to be diluted.

2. The serum may be diluted (*a*) by means of a graduated pipette—either a leucocytometer pipette (Fig. 46, *b*) or some corresponding form. In this way successive dilutions of 1 : 10, 1 : 20, 1 : 100, etc., can be rapidly made. This is the best method. (*b*) By means of a capillary pipette with a mark on the tube, the serum is drawn up to the mark and then blown out into a glass capsule; equal quantities of bouillon are successively measured in the same way

FIG. 46.—Tubes used in testing agglutinating and sedimenting properties of serum.

and added till the requisite dilution is obtained. (*c*) By means of a platinum needle with a loop at the end (Delépine's method). A loopful of serum is placed on a slide and the desired number of similar loopfuls of bouillon are separately placed around on the slide. The drops are then mixed.

A very convenient and rapid method of combining the steps 1 and 2



is to draw a drop of *blood* up to the mark 1 or .5 on a leucocytometer pipette and draw the bouillon after it till the bulb is filled. A dilution of 10 or 20 times is thus obtained. Then blow the mixture into a U-shaped tube (Fig. 46, *c*) and centrifugalise or simply allow the red corpuscles to separate by standing. (In this method of course the dilution is really greater than if pure serum were used, and allowance must therefore be made in comparing results.) The presence of red corpuscles is no drawback in the case of the microscopic method, but when sedimentation tubes are used the corpuscles should be separated first.

3. The bacteria to be tested should be taken from young cultures, preferably not more than twenty-four hours old, incubated at 37° C. They may be used either as a bouillon culture or as an emulsion made by adding a small portion of an agar culture to bouillon. In the latter case the mass of bacteria on a platinum loop should be gently broken down at the margin of the fluid in a watch-glass. When a thick turbidity is thus obtained, any remaining fragments should first be removed and then the organisms should be uniformly mixed with the rest of the fluid. The bacterial emulsion ought to have a faint but distinct turbidity. (When the exact degree of sedimenting power of a serum is to be tested—expressed as the highest dilution in which it produces complete sedimentation within twenty-four hours—a standard quantity (by weight) of bacteria must be added to a given quantity of bouillon. This is not necessary for clinical diagnosis.)

4. To test *microscopically*, mix equal quantities (measured by a marked capillary pipette) of the diluted serum and the bacterial emulsion on a glass slide, cover with a cover-glass, and examine under the microscope. The form of glass slide used for hang-drop cultures (Fig. 27) will be found very suitable. The ultimate dilution of the serum will, of course, be double the original dilution.

To observe *sedimentation* mix equal parts of diluted serum and of bacterial emulsion and place in a thin glass tube—a simple tube with closed end or a U-tube. Keep in upright position for twenty-four hours. One of Wright's sedimentation tubes is shown in Fig. 46, *d*. Diluted serum is drawn up to fill the space *mn*, a small quantity of air is sucked up after it to separate it from the bacterial emulsion, which is then drawn up in the same quantity; the diluted serum will then occupy the position *kl*. The fluids are then drawn several times up into the bulb and returned to the capillary tube so as to mix, and finally blown carefully down close to the lower end, which is then sealed off. The sediment collects at the lower extremity.

It may be said that it is often important to observe not only the strongest concentration of a serum which will produce agglutination but also the weakest.

**Method of measuring the Phagocytic Capacity of the Leucocytes—the Opsonic Technique.**—This was first done by Leishman by a very simple method as follows: A piece of quill tubing is drawn out to a capillary diameter so as to make a pipette about six inches long. The point is broken off and a rubber nipple adjusted to the wide end, a mark is made with an oil pencil about three-quarters of an inch above the orifice. Blood is drawn from the finger up to the mark, then an air-bubble is allowed to pass in. A thin

emulsion of the bacterium to be tested having been prepared, a quantity of this is also drawn up to the mark. The two fluids are then thoroughly mixed by being first blown out on to a sterile slide and then being drawn back into the pipette and expelled,—this being repeated several times. A cover-glass is placed over the drop, and the slide is placed in the incubator at 37° C. for fifteen minutes. The cover-glass is then slipped off so as to make a film preparation which in the case of ordinary bacteria may be stained by Leishman's method. The number of bacteria present in, say, 50 polymorphonuclear cells successively examined is determined and an average struck. The method was first used for showing that in cases of staphylococcus infection the average number of bacteria taken up was less than in a control in which the same bacterial emulsion was exposed to the blood of a healthy individual. In making such an observation drops from the two mixtures are placed on the same slide under separate cover-glasses and the preparation incubated. One cover is then slipped to one end of the slide and the other to the other,—the two films being then stained as one.

According to Wright's view the process of phagocytosis in blood outside the body is not a simple one, and before a leucocyte takes up a bacterium the latter must be acted on in some way by substances present in the serum, which Wright calls *opsonins* (see Immunity). The technique by which the actions of these opsonins is studied has been elaborated by Wright and his co-workers in connection with his work on bacterial vaccines, especially in relation to infection by the pyogenic cocci and the tubercle bacillus. This technique involves (1) the preparation of the bacterial emulsion, (2) the preparation of the leucocytes, (3) the preparation of samples of (a) serum from a normal person, (b) serum from the infected person.

(1) *Preparation of bacterial emulsion.* In the case of the pyogenic cocci a little of a twenty-four hour living culture off a sloped agar tube is taken and rubbed up in a watch-glass with .85 per cent saline. The mixture is placed in a tube and centrifugalised so as to deposit any masses of bacteria which may be present. Only by experience can a knowledge be gained of the amount of culture to be used in the first instance, but the resultant emulsion usually should exhibit only the merest trace of cloudiness to the naked eye. Wright states it will then contain from 7000 to 10,000 million bacteria per c.cm. If too strong an emulsion be used the leucocytes may take up so many organisms that these cannot be accurately enumerated. In the case of

the tubercle bacillus as short a variety of the organism as possible should be selected, and a mass of growth off a solid medium is taken (bacilli in mass can be obtained in the market from wholesale chemists) and is well washed with changes of distilled water, dried on filter paper in a Petri dish and thoroughly rubbed up with a little 1·5 per cent saline in an agate mortar so as to disintegrate the bacterial masses and get an emulsion composed as far as possible of individual bacilli. This must be controlled by microscopic examination. A thick cream should be obtained, and this should be sterilised by steaming for half an hour on three successive days. Before sterilisation it is convenient to seal up the stock emulsion in small quantities in a number of pieces of quill tubing so that in the subsequent procedures only small portions of the emulsion are exposed to aerial contamination at one time. For actual use, one of those tubes is opened, a little is withdrawn with a sterile pipette, and a weak emulsion made in the same way as with the staphylococcus except that 1·5 per cent saline is used. The stock tube may be sealed with wax and kept for use again. A fresh emulsion ought to be made up for each day's work.

(2) *Preparation of leucocytes.* Here the observer uses his own blood cells. A 1·5 per cent solution of sodium citrate in ·85 per cent sodium chloride is prepared. This is placed in a glass tube three inches long made by drawing out a piece of half-inch tubing to a point, the tube being filled nearly to the brim. A handkerchief being bound round the finger, this is now pricked and the blood allowed to flow directly into the fluid, to the bottom of which it sinks. The tube ought to be inverted between the addition of every few drops of blood so as to bring the blood in contact with the citrate and prevent coagulation. The equivalent of about ten to twenty drops of blood should be obtained. The diluted blood is then centrifugalised, and when the corpuscles are separated the supernatant fluid is removed, ·85 per cent saline is substituted and the centrifugalisation repeated. The fluid is again removed, care being taken not to disturb the layer of white cells lying on the top of the red corpuscles. This layer is then pipetted off into a watch-glass or tube and the leucocytes required are thus obtained.

(3) *Preparation of the sera.* The serum whose sensitising effect on the bacteria it is desired to test is obtained by Wright as follows. A "blood capsule" is made by drawing a piece of No. 3 quill tubing into the shape shown in Fig. 47, the part not drawn out being about one inch in length. It is convenient to

make a number of these capsules at one time and to draw off their extremities and seal them in the flame. For use the tips of both extremities are broken off, the finger is pricked, and blood allowed to pass into the capsule through the bent limb till the capsule is about half full. The air remaining in the capsule is rarefied by passing the straight end through a flame and then sealing it off. By this manipulation the blood is sucked over the bend into the straight part of the tube, and the bent end is now also sealed off or closed with wax. It is well to shake the blood down towards the closed straight end, care being taken to previously allow the glass to cool sufficiently. The capsule is now hung by the bend on the edge of a centrifuge tube and the serum separated by spinning the instrument. In

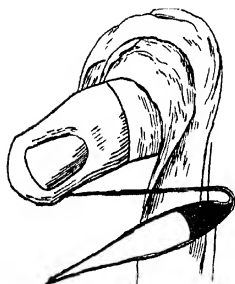


FIG. 47.—Wright's Blood-capsule and method of filling same.

any particular case a capsule of serum from the infected person and one from a normal individual are prepared.

The emulsion, corpuscles, and serum being thus prepared, the next step is to mix them. This is done by taking a piece of quill tubing and drawing it out to a capillary point so as to make a pipette about eight inches long; on the thick end of this a rubber teat is fixed, and about one inch from the capillary point a mark is made with an oil pencil. From the watch-glass containing the separated leucocytes a portion is sucked up to the mark and then an air-bubble is allowed to pass

in. A similar portion of the serum is drawn up, and then another air-bubble, and finally a similar portion of the bacterial emulsion. The three droplets are carefully blown on to a slide and are thoroughly mixed with one another by being alternately drawn up into the tube and expelled several times. The mixture is then drawn into the tube and the end sealed off in the flame. The rubber nipple is removed and the tube placed in the incubator at  $37^{\circ}$  for fifteen minutes. A slide is now prepared by rubbing it once or twice with very fine emery paper (No. 000) and thoroughly wiping it. This is a procedure adopted by Wright to cause an evenly distributed film to be made. The tube being removed from the incubator and the end broken off, its contents are again mixed by expelling and drawing up into the tube. A minute droplet is placed on the prepared slide, and by means of the edge of the end

of another slide a film is made which is then dried and is ready for staining. Films containing staphylococci are stained either by Leishman's stain (*q.v.*) or with carbol-thionin blue. In the former case no fixation is necessary, in the latter it is usual to fix in saturated perchloride of mercury for  $1\frac{1}{2}$  minutes, wash in water and then stain. With tubercle films the following is the procedure: the film is fixed for two minutes in perchloride of mercury, washed thoroughly, stained with carbol-fuchsin as usual, decolorised with 2.5 per cent sulphuric acid, cleared with 4 per cent acetic acid, counterstained with watery solution of methylene-blue, and dried.

In applying the technique two preparations are made, in both of which the same emulsion and the same leucocytes are employed, but in one of which the bacteria have been exposed to the serum of the infected individual under observation, and in the other to that of a normal person—usually the observer himself. Each of these is now examined microscopically with a movable stage, the number of bacteria in the protoplasm of at least 50 polymorphonucleated leucocytes is counted and an average per leucocyte struck; the proportion which this average in the case of the abnormal serum bears to the average in the preparation in which the healthy serum was used, constitutes the *opsonic index*,—that of the healthy serum being reckoned as unity. The reliability of the method of course depends on the phagocytic activity of the 50 cells counted representing the phagocytic activity of all the cells in the preparation.

#### GENERAL BACTERIOLOGICAL DIAGNOSIS.

Under this heading we have to consider the general routine which is to be observed by the bacteriologist when any material is submitted to him for examination. The object of such examination may be to determine whether any organisms are present, and if so, what organisms; or the bacteriologist may simply be asked whether a particular organism is or is not present. In any case his inquiry must consist (1) of a microscopic examination of the material submitted; (2) of an attempt to isolate the organisms present; and (3) of the identification of the organisms isolated. We must, however, before considering these points look at a matter often neglected by those who seek a bacteriological opinion, viz.: the *proper methods of obtaining and transferring to the bacteriologist the material which he is to be asked to examine*. The general principles here are (1) that every precaution must be adopted to prevent the material from



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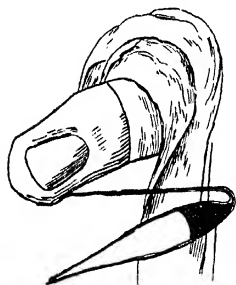


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being contaminated with extraneous organisms ; (2) that nothing be done which may kill any organisms which may be proper to the inquiry ; and (3) that the bacteriologist obtain the material as soon as possible after it has been removed from its natural surroundings.

The sources of materials to be examined, even in pathological bacteriology alone, are of course so varied that we can but mention a few examples. It is, for instance, often necessary to examine the contents of an abscess. Here the skin must be carefully purified by the usual surgical methods ; the knife used for the incision is preferably to be sterilised by boiling, the first part of the pus which escapes allowed to flow away (as it might be spoiled by containing some of the antiseptics used in the purification) and a little of what subsequently escapes allowed

to flow into a sterile test-tube. If test-tubes sterilised in a laboratory are not at hand, an ordinary test-tube may be a quarter filled with water, which is then well boiled over a spirit-lamp. The tube is then emptied and plugged with a plug of cotton wool, the outside of which has been singed in a flame. Small stoppered bottles may be sterilised and used in the same way. A discharge to be examined may be so small in quantity as to make the procedure described impracticable. It may be caught on a piece of sterile plain gauze, or of plain absorbent wool, which is then placed in a sterile vessel. Wool or gauze used for this purpose, or for swabbing out, say the throat, to obtain shreds of suspicious matter, must have no antiseptic impregnated in it, as the latter may kill the bacteria present and make the obtaining of cultures impossible.

FIG. 48.—Test-tube and pipette arranged for obtaining fluids containing bacteria.

Fluids from the body cavities, urine, etc., may be secured with sterile pipettes. To make one of these, take nine inches of ordinary quill glass-tubing, draw out one end to a capillary diameter, and place a little plug of cotton wool in the other end. Insert this tube through the cotton plug of an ordinary test-tube and sterilise by heat. To use it, remove test-tube plug with the quill tube in its centre, suck up some of the fluid into the latter, and replace in its former position in the test-tube (Fig. 48). Another method very convenient for transport is to make two constrictions on the glass tube at



suitable distances, according to the amount of fluid to be taken. The fluid is drawn up into the part between the constrictions, but so as not to fill it completely. The tube is then broken through at both constrictions and the thin ends are sealed by heating in a flame.

Solid organs to be examined should, if possible, be obtained whole. They may be treated in one of two ways. 1. The surface over one part about an inch broad is seared with a cautery heated to dull red heat. All superficial organisms are thus killed. An incision is made in this seared zone with a sterile scalpel, and small quantities of the juice are removed by a platinum spud to make cover-glass preparations and plate or smear cultures. 2. An alternative method is as follows:—The surface is sterilised by soaking it well with 1 to 1000 corrosive sublimate for half an hour. It is then dried, and the capsule of the organ is cut through with a sterile knife, the incision being further deepened by tearing. In this way a perfectly uncontaminated surface is obtained. Hints are often obtained from the clinical history of the case as to what the procedure ought to be in examination. Thus, as a matter of practice, cultures of tubercle and often of glanders bacilli can be easily obtained only by inoculation experiments. Typhoid bacilli need hardly be looked for in the faeces after the first ten days of the disease, and so on.

**Routine Procedure in Bacteriological Examination of Material.**—In the case of a discharge regarding which nothing is known the following procedure should be adopted:—(1) Several cover-glass preparations should be made. One ought to be stained with saturated watery methylene-blue, one with a stain containing a mordant such as Ziehl-Neelsen carbol-fuchsin, one by Gram's method. (2) (a) Gelatin plates should be made and kept at room temperature, (b) a series of agar plates or successive strokes on agar tubes (p. 55) should be made and incubated at 37° C. Method (b) of course gives results more quickly. If microscopic investigation reveals the presence of bacteria, it is well to keep the material in a cool place till next day when, if no growth has appeared in the incubated agar, some other culture medium (*e.g.* blood serum or agar smeared with blood) may be employed. If growth has taken place, say in the agar plates, one with about 200 or fewer colonies should be made the chief basis for research. In such a plate the first question to be cleared up is: Do all the colonies present consist of the same bacterium? The shape of the colony, its size, the appearance of the margin, the graining of the substance, its colour, etc., are all

to be noted. One precaution is necessary, viz., it must be noted whether the colony is on the surface of the medium or in its substance, as colonies of the same bacterium may exhibit differences according to their position. The arrangement of the bacteria in a surface colony may be still more minutely studied by means of *impression preparations*. A cover-glass is carefully cleaned and sterilised by passing quickly several times through a Bunsen flame. It is then placed on the surface of the medium and gently pressed down on the colony. The edge is then raised by a sterile needle, it is seized with forceps, dried high over the flame, and treated as an ordinary cover-glass preparation. In this way very characteristic appearances may sometimes be noted and preserved, as in the case of the anthrax bacillus. The colonies on a plate having been classified, a microscopic examination of each group may be made by means of cover-glass preparations, and tubes of gelatin and agar are inoculated from each representative colony. Each of the colonies used must be marked for future reference, preferably by drawing a circle round it on the under surface of the plate or capsule with one of Faber's pencils for marking on glass, a number or letter being added for easy reference.

The general lines along which observation is to be made in the case of a particular bacterium may be indicated as follows:—

1. *Microscopic Appearances*.—For ordinary descriptive purposes young cultures, say of 24 hours' growth, on agar should be used, though appearances in older cultures, such as involution forms, etc., may also require attention. Note (1) the form, (2) the size, (3) the appearance of the protoplasmic contents, especially as regards uniformity or irregularity of staining, (4) the method of grouping, (5) the staining reactions. Has it a capsule? Does the bacterium stain with simple watery solutions? Does it require the use of stains containing mordants? How does it behave towards Gram's method? It is important to investigate the first four points both when the organism is in the fluids or tissues of the body and when growing in artificial media, as slight variations occur. It must also be borne in mind that slight variations are observed according to the kind and consistence of the medium in which the organism is growing. (6) Is it motile and has it flagella? If so, how are they arranged? (7) Does it form spores, and if so, under what conditions as to temperature, etc.?

2. *Growth Characteristics*.—Here the most important points on which information is to be asked are, What are the characters of growth and what are the relations of growth (1) to

temperature, (2) to oxygen? These can be answered from some of the following experiments :—

A. Growth on gelatin. (1) Stab culture. Note (*a*) rate of growth; (*b*) form of growth, (*a*) on surface, (*β*) in substance; (*c*) presence or absence of liquefaction; (*d*) colour; (*e*) presence or absence of gas formation and of characteristic smell; (*f*) relation to reaction of medium. (2) Streak culture. (3) Shake culture. (4) Plate cultures. Note appearances of colonies (*a*) superficial, (*b*) deep. (5) Growth in fluid gelatin at 37° C.

B. Growth on agar at 37° C. (1) Stab. (2) Streak. Also on glycerin agar, blood agar, etc. Appearances of colonies in agar plates.

C. Growth in bouillon, (*a*) character of growth, (*b*) smell, (*c*) reaction.

D. Growth on special media. (1) Solidified blood serum. (2) Potatoes. (3) Lactose and other sugar media. Does fermentation occur and is gas formed? (4) Milk. Is it curdled or turned sour? (5) Litmus media. Note changes in colour. (6) Peptone solution. Is indol formed?

E. What is the viability of organism on artificial media?

3. *Results of inoculation experiments on animals.*

By attention to such points as these a considerable knowledge is attained regarding the bacterium, which will lead to its identification. In the case of many well-known organisms, however, a few of the above points taken together will often be sufficient for the recognition of the species, and experience teaches what are the essential points as regards any individual organism. In the course of the systematic description of the pathogenic organisms, it will be found that all the above points will be referred to, though not in every case.

The methods by which the morphological and biological characteristics of any growth may be observed have already been fully described. It need only be pointed out here that in giving descriptions of bacteria the greatest care must be taken to state every detail of investigation. Thus in any description of microscopic appearances the age of the growth from which the preparation was made, the medium employed, the temperature at which development took place must be noted, along with the stain which was used; and with regard to the latter it is always preferable to employ one of the well-known staining combinations, such as Löffler's methylene-blue. Especial care is necessary in stating the size of a bacterium. The apparent size often shows slight variations dependent on the stain used and the growth conditions of the culture. Accurate measurements of bacteria can only be made by preparing microphotographs of a definite magnification and measuring the sizes on the negatives. From these the actual sizes can easily be calculated. In describing bacterial cultures it must be borne in mind that the appearances

often vary with the age. It is suggested that in the case of cultures grown at from 36° to 37° C. the appearances between 24 and 48 hours should be made the basis of description, and in the case of cultures grown between 18° and 22° C. the appearances between 48 and 72 hours should be employed. The culture fluids used must be made up and neutralised by the precise methods already described. The investigator must give every detail of the methods he has employed in order that his observations may be capable of repetition.

### INOCULATION OF ANIMALS.<sup>1</sup>

The animals generally chosen for inoculation are the mouse, the rat, the guinea-pig, the rabbit, and the pigeon. Great caution must be shown in drawing conclusions from isolated experiments on rabbits, as these animals often manifest exceptional symptoms, and are very easily killed. Dogs are, as a rule, rather insusceptible to microbic disease, and the larger animals are too expensive for ordinary laboratory purposes. In the case of the mouse and rat the variety must be carefully noted, as there are differences in susceptibility between the wild and tame varieties, and between the white and brown varieties of the latter. In the case of the wild varieties, these must be kept in the laboratory for a week or two before use, as in captivity they are apt to die from very slight causes, and, further, each individual should be kept in a separate cage, as they show great tendencies to cannibalism. Of all the ordinary animals the most susceptible to microbic disease is the guinea-pig. Practically all inoculations are performed by means of the hypodermic syringe. The best variety is made on the ordinary model with metal mountings, asbestos washers, and preferably furnished with platinum-iridium needles. Before use the syringe and the needle are sterilised by boiling for five minutes. The materials used for inoculation are cultures, animal exudations, or the juice of organs. If the bacteria already exist in a fluid there is no difficulty. The syringe is most conveniently filled out of a shallow conical test-glass which ought previously to have been covered with a cover of filter paper and sterilised. If an inoculation is to be made from organisms growing on the surface of a solid medium, either a little ought to be scraped off and shaken up in sterile distilled water or .85 per cent salt solution to make an emulsion, or a little sterile fluid is poured on the growth and the latter scraped off into it. This fluid is then filtered into the test-glass through a plug of sterile glass wool. This is easily effected by taking a piece of  $\frac{5}{8}$  in. glass-tubing 3 in. long, drawing

<sup>1</sup> Experiments on animals, of course, cannot be performed in this country without a license granted by the Home Secretary.

one end out to a fairly narrow point, plugging the tube with glass wool above the point where the narrowing commences, and sterilising by heat. By filtering an emulsion through such a pipette, flocculi which might block the needle are removed. If a solid organ or an old culture is used for inoculation it ought to be rubbed up in a sterile porcelain or metal crucible with a little sterile distilled water, by means of a sterile glass rod, and the emulsion filtered as in the last case.

The methods of inoculation generally used are : (1) by scarification of the skin ; (2) by subcutaneous injection ; (3) by intraperitoneal injection ; (4) by intravenous injection ; (5) by injections into special regions, such as the anterior chamber of the eye, the substance of the lung, etc. Of these (2) and (3) are most frequently used. When an anaesthetic is to be administered, this is conveniently done by placing the animal, along with a piece of cotton-wool or sponge soaked in chloroform, under a bell-jar or inverted glass beaker of suitable size.

1. *Scarification*.—A few parallel scratches are made in the skin of the abdomen previously cleansed, just sufficiently deep to draw blood, and the infective material is rubbed in with a platinum eyelet. The disadvantage of this method is that the inoculation is easily contaminated. The method is only occasionally used.

2. *Subcutaneous Injection*.—A hypodermic syringe is charged with the fluid to be inoculated. The hair is cut off the part to be inoculated, and the skin purified with 1 to 1000 corrosive sublimate. The skin is then pinched up and, the needle being inserted, the requisite dose is administered. The wound is then sealed with a little collodion.

3. *Intraperitoneal Injection*.—This may be performed by means of a special form of needle. The needle is curved, and has its opening not at the point, but in the side in the middle of the arch (Fig. 49). The hair over the lower part of the abdomen is cut, and the skin purified with an antiseptic. The whole thickness of the abdominal walls is then pinched up by an assistant, between the forefingers and thumbs of the two hands. The needle is then plunged through the fold thus formed. The result is that the hole in the side of the needle is within the abdominal cavity, and the inoculation can thus be made. Intraperitoneal inoculation can also be practised with an

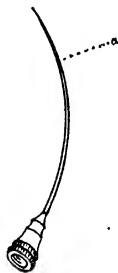


FIG. 49.—Hollow needle with lateral aperture (at *a*) for intraperitoneal inoculations.



ordinary needle. The mode of procedure is similar, but after the needle is plunged through the abdominal fold, it is partially withdrawn till the point is felt to be free in the peritoneal cavity when the injection is made. There is little risk of injuring the intestines by either method.

4. *Intravenous Injection*.—The vein most usually chosen is one of the auricular veins. The part has the hair removed, the skin is purified, and the vein made prominent by pressing on it between the point of inoculation and the heart. The needle is then plunged into the vein and the fluid injected. That it has perforated the vessel will be shown by the escape of a little blood; and that the injection has taken place into the lumen of the vessel will be known by the absence of the small swelling which occurs in subcutaneous injections. If preferred, the vein may be first laid bare by snipping the skin over it. The needle is then introduced.

5. *Inoculation into the Anterior Chamber of the Eye*.—Local anæsthesia is established by applying a few drops of 2 per cent solution of hydrochlorate of cocaine. The eye is fixed by pinching up the orbital conjunctiva with a pair of fine forceps, and the edge of the cornea being perforated by the hypodermic needle, the injection is easily accomplished.

Sometimes inoculations are made by planting small pieces of pathological tissues in the subcutaneous tissue. This is especially done in the case of glanders and tubercle. The skin over the back is purified, and the hair cut. A small incision is made with a sterile knife, and the skin being separated from the subjacent tissues by means of the ends of a blunt pair of forceps, a little pocket is formed into which a piece of the suspected tissue is inserted. The wound is then closed with a suture, and collodion is applied. In the case of guinea-pigs, the abdominal wall is to be preferred as the site of inoculation, as the skin over the back is extremely thick.

Injections are sometimes made into other parts of the body, *e.g.* the pleuræ and the cranium. It is unnecessary to describe these, as the application of the general principles employed above, together with those of modern aseptic surgery, will sufficiently guide the investigator as to the technique which is requisite.

After inoculation, the animals ought to be kept in comfortable cages, which must be capable of easy and thorough disinfection subsequently. For this purpose galvanised iron wire cages are the best. They can easily be sterilised by boiling them in the large fish-kettle which it is useful to have in a bacteriological laboratory for such a purpose. It is preferable to have the cages opening

from above. Otherwise material which may be infective may be scratched out of the cage by the animal. The general condition of the animal is to be observed, how far it differs from the normal, whether there is increased rapidity of breathing, etc. The temperature is usually to be taken. This is generally done *per rectum*. The thermometer (the ordinary 5 min. clinical variety) is smeared with vaselin, and the bulb inserted just within the sphincter, where it is allowed to remain for a minute; it is then pushed well into the rectum for five minutes. If this precaution be not adopted, a reflex contraction of the vessels may take place, which is likely to vitiate the result by giving too low a reading.

**Collodion Capsules.**—These have been used to allow the sojourn of bacteria within the animal body without their coming into contact with the cells of the tissues. Various substances in solution can pass in either direction through the wall by diffusion, but the wall is impermeable alike to bacteria and leucocytes. The following method of preparing such capsules is that of M'Rae modified by Harris. A gelatine capsule, such as is used by veterinary surgeons, is taken, and in one end there is fixed a small piece of thin glass tubing by gently heating the glass and inserting it. The tube becomes fixed when quite cold, and the junction is then painted round with collodion, which is allowed to dry thoroughly. The bore of the tubing is cleared of any obstructing gelatine, and the whole capsule is dipped into a solution of collodion so as to coat it completely. The collodion is allowed to dry and the coating is repeated; it is also advisable to strengthen the layer by further painting it at the extremity and at the junction. The interior of the capsule is then filled with water by a fine capillary pipette, and the capsule is placed in hot water in order to liquefy the gelatine, which can be removed from the interior by means of the fine pipette. The sac is filled with bouillon and is placed in a tube of bouillon. It is then sterilised in the autoclave. A small quantity of the bouillon is removed, and the contents are inoculated with the particular bacterium to be studied or an emulsion of the bacterium is added. The glass tubing is seized in sterile forceps and is sealed off in a small flame a short distance above the junction. The closed sac ought then to be placed in a tube of sterile bouillon to test its impermeability. The result is satisfactory if no growth occurs in the surrounding medium. The sac with its contents can now be transferred to the peritoneal cavity of an animal.

**Autopsies on Animals dead or killed after Inoculation.**—These should be made as soon as possible after death. It is

necessary to have some shallow troughs, constructed either of metal or of wood covered with metal, conveniently with sheet lead, and having a perforation at each corner to admit a tape or strong cord. The animal is tightly stretched out in the trough and tied in position. The size of the trough will therefore have to vary with the size of the outstretched body of the animal to be examined. In certain cases it is well to soak the surface of the animal in carbolic acid solution (1 to 20) or in corrosive sublimate (1 to 1000) before it is tied out. This not only to a certain extent disinfects the skin, but, what is more important, prevents hairs which might be affected with pathogenic products from getting into the air of the laboratory. The instruments necessary are scalpels (preferably with metal handles), dissecting forceps, and scissors. They are to be sterilised by boiling for five minutes. This is conveniently done in one of the small portable sterilisers used by surgeons. Two sets at least ought to be used in an autopsy, and they may be placed, after boiling, on a sterile glass plate covered by a bell-jar. It is also necessary to have a medium-sized hatchet-shaped cautery, or other similar piece of metal. It is well to have prepared a few freshly-drawn-out capillary tubes stored in a sterile cylindrical glass vessel, and also some larger sterile glass pipettes. The hair of the abdomen of the animal is removed. If some of the peritoneal fluid is wanted, a band should be cauterised down the linea alba from the sternum to the pubes, and another at right angles to the upper end of this; an incision should be made in the middle of these bands, and the abdominal walls thrown to each side. One or more capillary tubes should then be filled with the fluid collected in the flanks, the fluid being allowed to run up the tube and the point sealed off; or a larger quantity, if desired, is taken in a sterile pipette. If peritoneal fluid be not wanted, then an incision may be made from the episternum to the pubes, and the thorax and abdomen opened in the usual way. The organs ought to be removed with another set of instruments, and it is convenient to place them pending examination in deep Petri's capsules (sterile). It is generally advisable to make cultures and film preparations from the heart's blood. To do this, open the pericardium, sear the front of the right ventricle with a cautery, make an incision in the middle of the part seared, and remove some of the blood with a capillary tube for future examination, or, introducing a platinum eyelet, inoculate tubes and make cover-glass preparations at once. To examine any organ, sear the surface with a cautery, cut into it, and inoculate tubes and make film preparations with a platinum loop. For removing small parts of organs for making inoculations

on tubes, a small platinum spud is very useful, as the ordinary wires are apt to become bent. Place pieces of the organs in some preservative fluid for microscopic examination. The organs ought not to be touched with the fingers. When the examination is concluded the body should have corrosive sublimate or carbolic acid solution poured over it, and be forthwith burned. The dissecting trough and all the instruments ought to be boiled for half an hour. The amount of precaution to be taken will, of course, depend on the character of the bacterium under investigation, but as a general rule every care should be used.

## CHAPTER IV.

### BACTERIA IN AIR, SOIL, AND WATER.

#### ANTISEPTICS.

It is impossible here to do more than indicate the chief methods which are employed by bacteriologists in the investigation of the bacteria present in air, soil, and water, and to add an outline of the chief results obtained. In dealing with the latter the subject has been approached mainly from the standpoint of the bearings which the results have towards human pathology. In dealing with antiseptics, so far as possible the effects of the various agents on the chief pathogenic bacteria have been given, though in many cases our information is very imperfect.

#### AIR.

Very little information of value can be obtained from the examination of the air, but the following are the chief methods used, along with the results obtained. More can be learned from the examination of atmospheres experimentally contaminated than by the investigation of the air as it exists under natural conditions.

**Methods of Examination.**—The methods employed vary with the objects in view. If it be sought to compare the relative richness of different atmospheres in organisms, and if the atmospheres in question be fairly quiescent, then it is sufficient to expose gelatin plates for definite times in the rooms to be examined. Bacteria, or the particles of dust carrying them, fall on the plates, and from the number of colonies which develop a rough idea of the richness of the air in bacteria can be obtained. Petri states that in five minutes the bacteria present in 10 litres of air are deposited on 100 square centimetres of a gelatin plate.

More complete results are available when some method is employed by which the bacteria in a given quantity of air are examined. The oldest method employed, and one which is still used, is that of Hesse. The apparatus is shown in Fig. 50. It consists of a cylindrical tube *a* about 20 inches long and 2 inches in diameter. At one end this is closed by a rubber cork having a piece of quill tubing, *f*, passing through it and projecting some distance into the interior. For use the tube is sterilised in a tall "Koch," and then a quantity of peptone gelatin, sufficient to cover the whole interior to the thickness of an ordinary gelatin plate, is poured in. This gelatin is kept from escaping by the projection of the quill tubing into the lumen of the large tube. A plug of cotton wool is now placed in the outer end of the quill tubing. Over the other end of the large tube is tied a sheet of rubber having a hole about a quarter of an inch in diameter in its centre, and over this again is tied a piece of similar but unperforated sheet rubber. The tube is then sterilised in the tall "Koch." On removal from this it is rolled, after the manner of an Esmarch's tube (*q.v.*), till the gelatin is set as a layer over its interior, and it is then placed horizontally on the tripod as shown. The other part of the apparatus is an aspirator by means of which a known quantity of air can be brought in contact with the gelatin. It consists

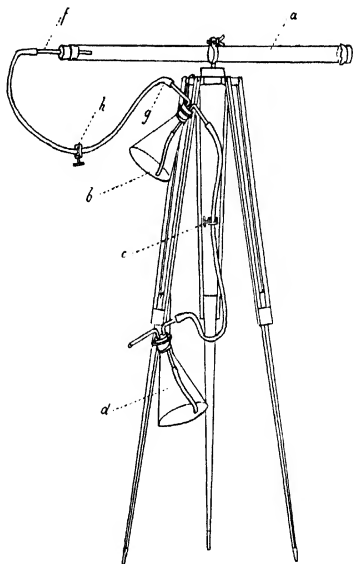


FIG. 50.—Hesse's tube, mounted for use.

of two conical glass flasks connected by means of a tube which passes through the cork of each down to the bottom of the flask. When this tube is filled with water, it, of course, can act as a syphon tube between volumes of water in the flasks. Such a syphon system being established, the levels of the water are marked on the flasks, and to one a litre of water is added, and by depressing flask *b* the whole litre can be got into it and the connecting tube *c* is then clamped. The two flasks are then connected by a rubber tube with the tube *f*, the clamp on *c* is opened, and the passing of a litre of water into *d* will draw a litre of air through the gelatin tube, when the outer rubber sheet is removed from the end and the clamp *h* opened. By disconnecting at *g* and reversing the syphon flasks, another litre can be sucked through, and so any desired quantity of air can be brought in contact with the gelatin. The speed ought not to be more than one litre in two minutes, and in such a case practically

all the organisms will be found to have fallen out of the air on to the gelatin in the course of their transit. This fact can be tested by interposing between the tube *a* and the aspirator a second tube prepared in the same way, which ought, of course, to show no growth. When forty-eight hours at 20° C. or four days at lower temperature have elapsed, the colonies which develop in *a* may be counted. The disadvantage of the method is that if particles of dust carrying more than one bacterium alight on the gelatin, these bacteria develop in one colony, and thus the enumeration results may be too low; difficulties may also arise from liquefying colonies developing in the upper parts of the tube and running over the gelatin.

**Petri's Sand-Filter Method.**—A glass tube open at both ends, and about 3½ inches long and half an inch wide, is taken, and in its centre is placed a transverse diaphragm of very fine iron gauze (Fig. 51, *e*); on each side of this is placed some fine quartz sand which has been well washed, dried, and burned to remove all impurities, and this is kept in position by cotton plugs. The whole is sterilised by dry heat. One plug is removed and a sterile rubber cork, *c*, inserted, through which a tube, *d*, passes to an exhausting apparatus. The tube is then clamped in an upright position in the atmosphere to be examined, with the remaining plug, *f*, uppermost. The latter is removed and the air sucked through. Difficulty may be experienced from the resistance of the sand if quick filtration be attempted. The best means to adopt is to use an air-pump—the amount of air drawn per stroke of which is accurately known—and have a manometer (as in Fig. 31) interposed between the tube and the pump. Between each two strokes of the air-pump the mercury is allowed to return to zero. After the required amount of air has passed, the sand *a* is removed, and is distributed among a number of sterile gelatin tubes which are well shaken; plate cultures are then made, and when growth has occurred the colonies are enumerated; the sand *b* is similarly treated and acts as a control.

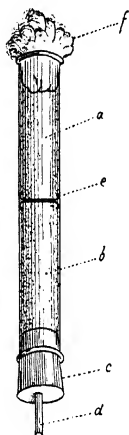


FIG. 51.—Petri's sand filter.

When it is necessary to examine air for particular organisms, special methods must often be adopted. Thus in the case of the suspected presence of tubercle bacilli a given quantity of air is drawn through a small quantity of water and then injected into a guinea-pig.

It must be admitted that comparatively little information bearing on the harmlessness or harmfulness of the air is obtainable by the mere enumeration of the living organisms present, for under certain conditions the number may be increased by the presence of many individuals of a purely non-pathogenic character. The organisms found in the air belong to two groups—firstly, a great variety of bacteria; secondly, yeasts and the spores of moulds and of the lower fungi. With regard to the spores, the organisms from which they are derived often

consist of felted masses of threads, from which are thrust into the air special filaments, and in connection with these the spores are formed. By currents of air these latter can easily be detached, and may float about in a free condition. With the bacteria, on the other hand, the case is different. Usually these are growing together in little masses on organic materials, or in fluids, and it is very much by the detachment of minute particles of the substratum that the organisms become free. The entrance of bacteria into the air, therefore, is associated with conditions which favour the presence of dust, minute droplets of fluid, etc. The presence of dust in particular would specially favour a large number of bacteria being observed, and this is the case with the air in many industrial conditions, where the bacteria, though numerous, may be quite innocuous. Great numbers of bacteria thus may not indicate any condition likely to injure health, and this may be true also even when the bacteria come from the crowding together of a number of healthy human beings. On the other hand, there is no doubt that disease germs can be disseminated by means of the air. The possibility of this has been shown experimentally by infecting the mouth with the *b. prodigiosus*, which is easily recognised by its brilliantly coloured colonies, and then studying its subsequent distribution. Most important here is the infection of the air from sick persons. The actions of coughing, sneezing, speaking, and even of deep breathing, distribute, often to a considerable distance, minute droplets of secretions from the mouth, throat, and nose, and these may float in the air for a considerable time. Even five hours after an atmosphere has been thus infected evidence may be found of bacteria still floating free. Before this time, however, most of the bacteria have settled upon various objects, where they rapidly dry, and are no longer displaceable by ordinary air currents. The diseases of known etiology where infection can thus take place are diphtheria, influenza, pneumonia, and phthisis; and here also probably whooping-cough, typhus fever, and measles are to be added, though the morbid agents are unknown. In the case of phthisis, the alighting of tubercle bacilli has been demonstrated on cover-glasses held before the mouths of patients while talking, and animals made to breathe directly in front of the mouths of such patients have become infected with tuberculosis. Apart from direct infection from individuals, however, pathogenic bacteria may be spread in some cases from the splashing of infected water, as from a sewage outfall. This possibility has to be recognised especially in the cases of typhoid and cholera. Besides infection through fluid particles,



infection can be caused in the air by dust coming, say, from infected skin or clothes, etc. Flügge, in dealing with this subject in an experimental inquiry, distinguishes between large particles of dust which require an air current moving at the rate of 1 centimetre per second to keep them suspended, and the finer dust which can be kept in suspension by currents moving at from 1 to 4 millimetres per second. In the former case, when once the particles alight they cannot be displaced by currents of air except when these are moving at, at least, 5 metres per second, but the brushing, shaking, or beating of objects may, of course, distribute them. In the case of the finer dust the particles will remain for long suspended, and when they have settled can be more easily displaced, as by the waving of an arm, breathing, etc. With regard to infection by dust, a most important factor, however, is whether or not the infecting agent can preserve its vitality in a dry condition. In the case of a sporing organism such as anthrax, vitality is preserved for long periods of time, and great resistance to drying is also possessed by the tubercle and diphtheria bacilli; but apart from such cases there is little doubt that infection is usually necessarily associated with the transport of moist particles, and is thus confined to a limited area around a sick person. Among diseases which may occasionally be thus spread cholera and typhoid have been classed. Considerable controversy has arisen with regard to certain outbreaks of the latter disease, which have apparently been spread by dusty winds, although we have the fact that the typhoid bacillus does not survive being dried even for a short time. It appears, however, that in such epidemics the transport of infection by means of insects carried by the wind has not been entirely excluded.

As in the cases of the soil and of water, presently to be described, attempts have been made to obtain indirect evidence of the contamination of the air by man. Thus Gordon has shown that certain streptococci are common in the saliva; these resemble the streptococcus pyogenes, but are relatively non-pathogenic, grow well at 37° C. and under anaerobic conditions, cause clotting and acid-formation in litmus milk at 37°, and in neutral-red media have an action resembling that of *b. coli*. These characters serve, according to Gordon, to differentiate organisms of human origin from ordinary streptococci occurring in the air and which he states grow better at about 22° C., are facultative anaerobes and do not produce the changes in milk and in neutral-red media. Thus the finding of streptococci of the first group in plates exposed to air would indicate that a human source was probable, and, if the observation were made on air from the neighbourhood of a sick person, that risk of the dissemination of disease germs was present. The value of this as a practical method has yet to be determined.

## SOIL.

The investigation of the bacteria which may be found in the soil is undertaken from various points of view. Information may be desired as to the change its composition undergoes by a bacterial action, the result of which may be an increase in fertility and thus in economic value. Under this head may be grouped inquiries relating to the bacteria which convert ammonia and its salts into nitrates and nitrites, and to the organisms concerned in the fixation of the free nitrogen of the air. The discussion of the questions involved in such inquiries is outside the scope of the present chapter, which is more concerned with the relation of the bacteriology of the soil to questions of public health. So far as this narrower view is concerned, soil bacteria are chiefly of importance in so far as they can be washed out of the soils into potable water supplies. An important aspect of this question thus is as to the significance of certain bacteriological appearances in a water in relation to the soil from which it has come or over which it has flowed. In this country these questions have been chiefly investigated by Houston, and it is from his papers that the following account is largely taken.

**Methods of Examination.**—For examination of soil on surface or not far from surface, Houston recommends tin troughs 10 in. by 3 in. and pointed at one extremity, to be wrapped in layers of paper and sterilised by dry heat. If several of these be provided, then the soil can be well rubbed up and a sample secured and placed in a sterile test-tube for examination as soon as convenient after collection. If samples are to be taken at some depth beneath the surface, then a special instrument of which many varieties have been devised must be used. The general form of these is that of a gigantic gimlet stoutly made of steel. Just above the point of the instrument the shaft has in it a hollow chamber, and a sliding lateral door in this can be opened and shut by a mechanism controlled at the handle. The chamber being sterilised and closed, the instrument is bored to the required depth, the door is slid back, and by varying devices it is effected that the chamber is filled with earth; the door is reclosed and the instrument withdrawn.

In any soil the two important lines of inquiry are first as to the total number of organisms (usually reckoned per gramme of the fresh sample), and secondly as to the varieties of organisms present. The number of organisms present in a soil is often, however, so enormous that it is convenient to submit only a fraction of a gramme to examination. The method employed is to weigh the tube containing the soil, shake out an amount of about the size of a bean into a litre of distilled water, and reweigh the tube. The amount placed in the water is distributed as thoroughly as possible by shaking, and, if necessary, by rubbing down with a sterile glass rod, and small quantities measured from a graduated pipette are used for the investigation. For estimating the total number of organisms present in the portion of soil used, small quantities, say .1 c.c. and 1 c.c., of the fluid are added to melted tubes of ordinary alkaline peptone gelatin; after being shaken, the gelatin is plated, incubated at

22° C., and the colonies are counted as late as the liquefaction, which always occurs round some of them, will allow. From these numbers the total number of organisms present in the amount of soil originally present can be calculated.

The numbers of bacteria in the soil vary very much. According to Houston's results, fewest occur in uncultivated sandy soils, these containing on an average 100,000 per gramme. Peaty soils, though rich in organic matter, also give low results, it being possible that the acidity of such soils inhibits free bacterial growth. Garden soils yield usually about 1,500,000 bacteria per gramme, but the greatest numbers are found in soils which have been polluted by sewage, when the figures may rise to 115,000,000. In addition to the enumeration of the numbers of bacteria present, it is a question whether something may not be gained from a knowledge of the number of spores present in a soil relative to the total number of bacteria. This is a point which demands further inquiry, especially by the periodic investigation of examples of different classes of soils. The method is to take 1 c.c. of such a soil emulsion as that just described, add it to 10 c.c. of gelatin, heat for ten minutes at 80° C. to destroy the non-spored bacteria, plate, incubate, and count as before.

Besides the enumeration of the numbers of bacteria present in a soil, an important question in its bacteriological examination lies in inquiring what kinds of bacteria are present in any particular case. Practically this resolves itself into studying the most common bacteria present, for the complete examination of the bacterial flora of any one sample would occupy far too much time. Of these common bacteria the most important are those from whose presence indications can be gathered of the contamination of the soil by sewage, for from the public health standpoint this is by far the most important question on which bacteriology can shed light.

*Bacillus mycoides*.—This bacillus is 1.6 to 2.4  $\mu$  in length and about .9 in breadth. It grows in long threads which often show motility. It can be readily stained by such a combination as carbol-thionin, and retains the dye in Gram's method. All ordinary media will support its growth, and, in surface growths on agar or potato spore, formation is readily produced. Its optimum temperature is about 18° C. On gelatin plates it shows a very characteristic appearance. At first under a low power it shows a felted mass of filaments throwing out irregular shoots from the centre, and later to the naked eye these appear to be in the form of thick threads like the growth of a mould. They rapidly spread over the surface of the medium, and the whole resembles a piece of wet teased-out cotton wool. The gelatin is liquefied.

*Cladothrices*.—Of these several kinds are common in the soil. The ordinary *cladothrix dichotoma* is among them. This organism appears

as colourless flocculent growth with an opaque centre, and can be seen under the microscope to send out into the medium apparently branched threads which vary in thickness, being sometimes  $2\mu$  across. They consist of rods enclosed in a sheath. These rods may divide at any point, and thus the terminal elements may be pushed along the sheath. Sometimes the sheath ruptures, and thus by the extrusion of these dividing cells and their further division the branching appearance is originated. Reproduction takes place by the formation of gonidia in the interior of the terminal cells. These gonidia acquire at one end a bundle of flagella, and for some time swim free before becoming attached and forming a new colony. Houston describes as occurring in the soil another variety, which with similar microscopic characters appears as a brownish growth with a pitted surface, and diffusing a bismarck-brown pigment into the gelatin which it liquefies.

A few experiments made with an ordinary field soil will, however, familiarise the worker with the non-pathogenic bacteria usually present. We have referred to these two because of their importance. In regard to pathogenic organisms, especially in relation to possible sewage contamination, attention is to be directed to three groups of organisms, those resembling the *b. coli*, the *Bacillus enteritidis sporogenes*, and the *Streptococcus pyogenes*. The characters of the first two of these will be found in the chapter on Typhoid Fever; of the third in Chap. VI. For the detection of these bacteria Houston recommends the following procedure.

(a) The *B. coli* group. A third of a gramme of soil is added to 10 c.c. phenol broth (*vide* chapter on Typhoid Fever) and incubated at  $37^{\circ}\text{C}$ . In this medium very few if any other bacteria except those of the *b. coli* group will grow, so that if after twenty-four hours a turbidity appears, some of the latter may be suspected to be present. In such a case a loopful of the broth is shaken up in 5 c.c. sterile distilled water, and of this one or two loopfuls are spread over the surface of a solid plate of phenol gelatin in a Petri capsule either by means of the loop or of a small platinum spatula, and the plate is incubated at  $20^{\circ}\text{C}$ . Any colonies which resemble *b. coli* are then examined by the culture methods detailed under that organism. Further, all organisms having the microscopic appearances of *b. coli*, and which generally conform to its culture reactions, are to be reckoned in the *coli* group. The media of MacConkey and Drigalski are very useful in connection with the separation of such soil organisms (*v. pp.* 42, 43).

(b) The *Bacillus enteritidis sporogenes*. To search for this organism 1 gramme of the soil is thoroughly distributed in 100 c.c. sterile distilled water, and of this 1 c.c., .1 c.c., and .01 c.c. is added to each of three sterile milk tubes. These are heated to  $80^{\circ}\text{C}$ . for ten minutes and then cultivated anaerobically at  $37^{\circ}\text{C}$ . for twenty-four hours. If the characteristic appearances seen in such cultures of the *b. enteritidis (q.v.)* are developed, then it may fairly safely be deduced that it is this organism which has produced them.

(c) *Fæcal Streptococci*. The method here is to pour out a tube of agar into a Petri capsule, and when it has solidified to spread out .1 c.c. of the emulsion of soil over it and incubate at  $37^{\circ}\text{C}$ . for twenty-four hours. At this temperature many of the non-pathogenic bacteria grow with difficulty, and thus the number of colonies which develop is relatively small. Colonies having appearances resembling those of the *Streptococcus pyogenes (q.v.)* can thus be investigated. Much work has been devoted to the question of these fæcal streptococci presenting specific characters by

stopper with forceps. Care must be taken not to touch the water-bed, as the vegetable matter covering it contains a large number of organisms. The bottles ought to be packed in ice and sawdust, and plates must be prepared from the samples as soon as possible. When the object in view is to determine the number of bacteria per cubic centimetre, it is important to note that water bacteria grow at very varied rates, and therefore it is well that the same time should always elapse before the colonies are counted. The period of growing usually allowed is forty-eight hours at 20° C.

Several points may be here noted. It has been found, for instance, that slight variations in the reaction of the medium affect the number of colonies which develop. A slightly greater degree of alkalinity than peptone gelatin, as ordinarily prepared, possesses—such an increased degree as that caused by the addition of .01 grm.  $\text{Na}_2\text{CO}_3$  to 10 c.c. peptone gelatin—will give a greater yield of colonies than the ordinary gelatin. Again, the natural temperature of the growth of water bacteria in temperate climates is comparatively low, being not often above 18° C., and, on account of this, gelatin suggests itself as the most suitable medium. This can be seen by comparing the growth on an agar plate inoculated with a given quantity of water, and incubated at 37° C., with the growth on a precisely similar gelatin plate incubated at 20° C., as it will be found that many more colonies have developed on the latter. This fact may be taken advantage of when pathogenic bacteria are being sought for in a water. The latter usually grow well at 37° C., and thus if agar plates be used the search may be facilitated. Apart from the difference of incubation temperatures, however, in such a case as that cited, it is probable that agar is a less suitable medium than gelatin for the growth of water bacteria, for in plates incubated at the same temperature the colonies which grow on the agar are often fewer than those on the gelatin. Probably no one medium will support the growth of all the organisms present in a given sample of water, and under certain circumstances special media must therefore be used. Thus Hansen found that in testing waters to be used in brewing it was advisable to have in the medium employed some sterile wort or beer, so that the organisms in the test experiments should be provided with the food materials which would be present in the commercial use of the water. Manifestly this principle applies generally in the bacteriological examination of waters to be used for industrial purposes.

In ordinary public health work it may be taken that the most frequent and important inquiry is directed towards the presence or absence of the *b. coli* and its congeners. Many methods are here used but we consider that in which MacConkey's bile-salt media are employed the most convenient. For small quantities of water,—up to 1 c.c.,—the sample is simply added to a Durham's tube of bile-salt glucose neutral-red broth and incubated for 48 hours. When it is necessary to examine larger samples it is convenient, as Savage recommends, to have the bile-salt broth made of double, treble, or quadruple its usual strength. The water to be examined is used as the diluent by which the medium is brought down to the ordinary concentration. If gas forms, some of the *coli* group are almost certainly present. The organisms may be plated out by smearing a little of the broth on bile-salt agar for further isolation and examination.

With regard to the objects with which the bacteriological

examination of water may be undertaken, though these may be of a purely scientific character, they usually aim at contributing to the settlement of questions relating to the potability of waters, to their use in commerce, and to the efficiency of processes undertaken for the purification of waters which have undergone pollution. The last of these objects is often closely associated with the first two, as the question so often arises whether a purification process is so efficient as to make the water again fit for use.

Water derived from any natural source contains bacteria, though, as in the case of some artesian wells and some springs, the numbers may be very small, *e.g.* 4 to 100 per c.c. In rain, snow, and ice there are often great numbers, those in the first two being derived from the air. Great attention has been paid to the bacterial content of wells and rivers. With regard to the former, precautions are necessary in arriving at a judgment. If the water in a well has been standing for some time, multiplication of bacteria may give a high value. To meet this difficulty, if practicable, the well ought to be pumped dry and then allowed to fill, in order to get at what is really the important point, namely, the bacterial content of the water entering the well. Again, if the sediment of the well has been stirred up a high value is obtained. Ordinary wells of medium depth contain from 100 to 2000 per c.c. With regard to rivers very varied results are obtained. Moorland streams are usually very pure. In an ordinary river the numbers present vary at different seasons of the year, whilst the prevailing temperature, the presence or absence of decaying vegetation, or of washings from land, and dilution with large quantities of pure spring water, are other important features. Thus the Franklands found the rivers Thames and Lea purest in summer, and this they attributed to the fact that in this season there is most spring water entering, and very little water as washings off land. In the case of other rivers the bacteria have been found to be fewest in winter. A great many circumstances must therefore be taken into account in dealing with mere enumerations of water bacteria, and such enumerations are only useful when they are taken simultaneously over a stretch of river, with special reference to the sources of the water entering the river. Thus it is usually found that immediately below a sewage effluent the bacterial content rises, though in a comparatively short distance the numbers may markedly decrease, and it may be that the river as far as numbers are concerned may appear to return to its previous bacterial content. The numbers of

are required, though on this point there may be some difference of opinion. Certainly very fair results are obtained when apparently the conditions chiefly favour aerobic organisms alone. This is usually effected by running the sewage on to beds of sand, or preferably of coke, allowing it to stand for some hours, slowly running the effluent out through the bottom of the bed, and leaving the bed to rest for some hours before recharging. The final result is better if the effluent be afterwards run over another similar coke-bed. According to some authorities the sewage, as it runs into the first bed, takes up from the air considerable free oxygen, which, however, soon disappears during the stationary period, so that on leaving the first bed the sewage contains little oxygen. In the latter part of its stay it has thus been submitted to anaerobic conditions. Further, while by the passage of the effluent out of the first bed oxygen is sucked in, this rapidly disappears, and during the greater part of the resting stage the interstices of the bed are filled with carbonic acid gas, with nitrogen partly derived from the air, partly from putrefactive processes, and thus in the filter anaerobic conditions prevail, under which the bacteria can act on the deposit left on the coke. On this latter point there is difference of opinion, for, in examining London sewage, Clowes has found oxygen present in abundance from four to forty hours after the sewage has been run off. Sometimes the treatment of the sewage consists in allowing it continuously to trickle through sand or gravel or coke beds. Probably the best results in sewage treatment are obtained when it is practicable to introduce a step where there can be no doubt that the conditions are anaerobic. This involves as a preliminary stage the treatment of the sewage in what is called a septic tank, and the method has been adopted at Exeter, Sutton, and Yeovil in this country, and very fully worked at in America by the State Board of Health of Massachusetts. In the explanation given of the rationale of this process, sewage is looked on as existing in three stages. (1) First of all, *fresh sewage*—the newly mixed and very varied material as it enters the main sewers. (2) Secondly, *stale sewage*—the ordinary contents of the main sewers. Here there is abundant oxygen, and as the sewage flows along there occurs by bacterial action a certain formation of carbon dioxide and ammonia which combine to form ammonium carbonate. This is the sewage as it reaches the purification works. Here a preliminary mechanical screening may be adopted, after which it is run into an air-tight tank—the septic tank. (3) It remains there for from twenty-four to thirty-six hours, and becomes a foul-smelling fluid—the *septic sewage*. The chemical

changes which take place in the septic tank are of a most complex nature. The sewage entering it contains little free oxygen, and therefore the bacteria in the tank are probably largely anaerobic, and the changes which they originate consist of the formation of comparatively simple compounds of hydrogen with carbon, sulphur, and phosphorus. As a result there is a great reduction in the amount of organic nitrogen, of albuminoid ammonia, and of carbonaceous matter. The latter fact is important, as the clogging of ordinary filter beds is largely due to the accumulation of such material, and of matters generally consisting of cellulose. One further important effect is that the size of the deposited matter is decreased, and therefore it is more easily broken up in the next stage of the process. This consists of running the effluent from the septic tank on to filter beds, preferably of coke, where a further purification process takes place. By this method there is first an anaerobic treatment succeeded by an aerobic; in the latter the process of nitrification occurs by means of the special bacteria concerned. The results are of a satisfactory nature, there being often a marked diminution in the number of coli organisms present.

Often the effluent from a sewage purification system contains as many bacteria as the sewage entering, but, especially by means of the septic tank method, there is often a marked diminution. It is said by some that pathogenic bacteria do not live in sewage. The typhoid bacillus has been found to die out when placed in sewage, but it certainly can live in this fluid for a much longer period than that embraced by any purification method. Thus the constant presence of *b. coli*, *b. enteritidis*, and streptococci which has been observed in sewage effluents must here still be looked on as indicating a possible infection with the typhoid bacillus, and it is only by great dilution and prolonged exposure to the conditions present in running water that such an effluent can be again a part of a potable water.

#### ANTISEPTICS.

The death of bacteria is judged of by the fact that when they are placed on a suitable food medium no development takes place. Microscopically it would be observed that division no longer occurred, and that in the case of motile species movement would have ceased, but such an observation has only scientific interest. From the importance of being able to kill bacteria an enormous amount of work has been done in the way of investigating the means of doing so by chemical means,



and the bodies having such a capacity are called antiseptics. It is now known that the activity of these agents is limited to the killing of bacteria outside the animal body, but still even this is of high importance.

**Methods.**—These vary very much. In early inquiries a great point was made of the prevention of putrefaction, and work was done in the way of finding how much of an agent must be added to a given solution such as beef extract, urine, etc., in order that the bacteria accidentally present might not develop; but as bacteria vary in their powers of resistance, the method was unsatisfactory, and now an antiseptic is usually judged of by its effects on pure cultures of definite pathogenic microbes, and in the case of a sporing bacterium the effect on both the vegetative and spore forms is investigated. The organisms most used are the *staphylococcus pyogenes*, *streptococcus pyogenes*, and the organisms of typhoid, cholera, diphtheria, and anthrax—the latter being most used for testing the action on spores. The best method to employ is to take sloped agar cultures of the test organism, scrape off the growth, and mix it up with a small amount of distilled water and filter this emulsion through a plug of sterile glass wool held in a small sterile glass funnel, add a measured quantity of this fluid to a given quantity of a solution of the antiseptic in distilled water, then after the lapse of the period of observation to remove one or two loopfuls of the mixture and place them in a great excess of culture medium. Here it is preferable to use fluid agar, which is then plated and incubated; such a procedure is preferable to the use of bouillon tubes, as any colonies developing can easily be recognised as belonging to the species of bacterium used. In dealing with strong solutions of chemical agents it is necessary to be sure that the culture fluid is in great excess, so that the small amount of the antiseptic which is transferred with the bacteria may be diluted far beyond the strength at which it still can have any noxious influence. Sometimes it is possible at the end of the period of observation to change the antiseptic into inert bodies by the addition of some other substance and then test the condition of the bacteria, and if the inert substances are fluid there is no objection to this proceeding, but if in the process a precipitate results, then it is better not to have recourse to such a method, as sometimes the bacteria are carried down with the precipitate and may escape the culture test. The advisability of, when possible, thus chemically changing the antiseptic was first brought to notice by the criticism of Koch's statements as to the efficacy of mercuric chloride in killing the spores of the *b. anthracis*. The method he employed in his experiments was to soak silk threads in an emulsion of anthrax spores and dry them. These were then subjected to the action of the antiseptic, well washed in water, and laid on the surface of agar. It was found, however, that with threads exposed to a far higher concentration of the corrosive sublimate than Koch had stated was sufficient to prevent growth, if the salt were broken up by the action of ammonium sulphide and this washed off, growth of anthrax still occurred when the threads were laid on agar. The explanation given was that the antiseptic had formed an albuminate with the case of each spore, and that this prevented the antiseptic from acting upon the contained protoplasm. Such an occurrence only takes place with spores, and the method given above, in which the small amount of antiseptic adhering

to the bacteria is swamped in an excess of culture fluid, can safely be followed, especially when a series of antiseptics is being compared.

Much attention has been paid to the standardisation of antiseptics, and a watery solution of carbolic acid is now generally taken as the standard with which other antiseptics are compared. Rideal and Walker point out that 110 parts by weight of B.P. carbolic acid equal 100 parts by weight of phenol, and they recommend the following method of standardising. To 5 c.c. of a particular dilution of the disinfectant add 5 drops of a 24-hour-old bouillon culture of the organism (usually *b. typhosus*) which has been incubated at 37° C. Shake the mixture and make subcultures every 2½ minutes to 15 minutes. Perform a parallel series of experiments with carbolic acid and express the comparative result in multiples of the carbolic acid doing the same work.

**The Action of Antiseptics.**—In inquiries into the actions of antiseptics attention to a great variety of factors is necessary, especially when the object is not to compare different antiseptics with one another, but when the absolute value of any body is being investigated. Thus the medium in which the bacteria to be killed are situated, is important; the more albuminous the surroundings are, the greater degree of concentration is required. Again, the higher the temperature at which the action is to take place, the more dilute may the antiseptic be, or the shorter the exposure necessary for a given effect to take place. The most important factor, however, to be considered is the chemical nature of the substances employed. Though nearly every substance which is not a food to the animal or vegetable body is more or less harmful to bacterial life, yet certain bodies have a more marked action than others. Thus it may be said that the most important antiseptics are the salts of the heavy metals, certain acids, especially mineral acids, certain oxidising and reducing agents, a great variety of substances belonging to the aromatic series, and volatile oils generally. In comparing different bodies belonging to any one of these groups the chemical composition or constitution is very important, and if such comparisons are to be made, the solutions compared must be equimolecular; in other words, the action of a molecule of one body must be compared with the action of a molecule of another body. This can be done by dissolving the molecular weight in grammes in say a litre of water (see p. 33). When this is done important facts emerge. Thus, generally speaking, the compounds of a metal of high atomic weight are more powerful antiseptics than those of one belonging to the same series, but of a lower atomic weight. Among organic bodies again substances with high molecular weight are more powerful than those of low molecular weight—thus butyric alcohol is more powerful than ethylic alcohol—and important differences among

the aromatic bodies are associated with their chemical constitution. Thus among the cresols the ortho- and para-bodies resemble each other in general chemical properties, and stand apart from metacresol ; they also are similar in antiseptic action, and are much stronger than the meta-body. The same may be observed in the other groups of ortho-, meta-, and para-bodies. Again, such a property as acidity is important in the action of a substance, and, generally speaking, the greater the avidity of an acid to combine with an alkali, the more powerful an antiseptic it is. With regard to oxidising agents and reducing agents, probably the possession of such properties has been overrated as increasing bactericidal potency. Thus in the case of such reducers as sulphurous acid and formic acid, the effect is apparently chiefly due to the fact that these substances are acids. Formic acid is much more efficient than formate of sodium. In the case of permanganate of potassium, which is usually taken as the type of oxidising agents in this connection, it can be shown that the greater amount of the oxidation which takes place when this agent is brought into contact with bacteria occurs after the organisms are killed. Such an observation is, however, not conclusive as to the non-efficiency of the oxidation process, for the death of the bacteria might be due to the oxidation of a very small part of the bacterial protoplasm. Apart from the chemical nature of antiseptic agents, the physical factors concerned in their solution, especially when they are electrolytes, probably play a part in their action. The part played by such factors is exemplified in the important fact that a strong solution acting for a short time will have the same effect as a weaker solution acting for a longer time. From what has been said it will be realised that the real causes of a material being an antiseptic are very obscure, and at present we can only have a remote idea of the factors at work.

**The Actions of certain Antiseptics.**—Here we can only briefly indicate certain results obtained with the more common members of the group.

*Chlorine.*—All the halogens have been found to be powerful antiseptics, but from the cheapness with which it can be produced chlorine has been most used ; not only is it the chief active agent in the somewhat complex action of bleaching powder, but it is also the chief constituent of several proprietary substances, of which "Electrozone" is a good example. This last substance is made from electrolysing sea-water, when magnesia and chlorine being liberated, magnesium hypochlorite and magnesium chloride are formed. In the action of this substance free hypo-

chlorous acid is formed, and the effect produced is thus similar to that of bleaching powder. Nissen, investigating the action of the latter, found that  $1\frac{1}{2}$  per cent killed typhoid bacilli in fæces; and Rideal found that 1 part to 400-500 disinfected sewage in fourteen minutes, and Delépine's results show that 1 part to 50 (equal to .66 per cent of chlorine) rapidly kills the tubercle bacillus, and 1 part to 10 (equal to 3.3 per cent) killed anthrax spores. Klein found that .05 per cent of chlorine killed most bacterial spores in five minutes.

*Iodine Terchloride.*—This is a very unstable compound of iodine and chlorine, and though it has been much used as an antiseptic, seeing that the substance only remains as  $\text{ICl}_3$  in an atmosphere of chlorine gas, it is open to doubt whether the effects described are not due to a very complicated action of free hydrochloric acid, hydriodic acid, of oxyacids of chlorine and iodine produced by its decomposition, and also, in certain cases, of organic iodine compounds formed from its contact with albuminous material. It is stated that the action is very potent: a 1 per cent solution is said instantly to kill even anthrax spores, but if the spores be in bouillon, death occurs after from ten to twelve minutes. In serum the necessary exposure is from thirty to forty minutes. A solution of 1-1000 will kill the typhoid, cholera, and diphtheria organisms in five minutes.

*Nascent Oxygen.*—This is chiefly available in two ways—firstly, when in the breaking up of ozone the free third atom of the ozone molecule is seeking to unite with another similar atom; secondly, when peroxide of hydrogen is broken up into water and an oxygen atom is thereby liberated. In commerce the activity of "Sanitas" compounds is due to the formation of ozone by the slow oxidation of the resin, camphor, and thymol they contain.

*Perchloride of Mercury.*—Of all the salts of the heavy metals this has been most widely employed, and must be regarded as one of the most powerful and useful of known antiseptics. In testing its action on anthrax spores there is no doubt that in the earlier results its potency was overrated from a neglect of the fact already alluded to, that in the spore-case an albuminate of mercury was formed which prevented the contained protoplasm from developing, while not depriving it of life. It has been found, however, that this salt in a strength of 1-100 will kill the spores in twenty minutes, although an hour's exposure to 1-1000 has no effect. The best results are obtained by the addition to the corrosive sublimate solution of .5 per cent of sulphuric acid or hydrochloric acid; the spores will then be killed by a seventy-

minute exposure to a 1-200 solution. When, however, organisms in the vegetative condition are being dealt with, much weaker solutions are sufficient; thus anthrax bacilli in blood will be killed in a few minutes by 1-2000, in bouillon by 1-40,000, and in water by 1-500,000. Plague bacilli are killed by one to two minutes' exposure to 1-3000. Generally speaking, it may be said that a 1-2000 solution must be used for the practically instantaneous killing of vegetative organisms.

Perchloride of mercury is one of the substances which have been used for disinfecting rooms by distributing it from a spray producer, of which the Equifex may be taken as a type. With such a machine it is calculated that 1 oz. of perchloride of mercury used in a solution of 1-1000 will probably disinfect 3000 square feet of surface. Such a procedure has been extensively used in the disinfection of plague houses, but the use of a stronger solution (1-500 acidulated) is probably preferable.

*Formalin* as a commercial article is a 40 per cent solution of formaldehyde in water. This is a substance which of late years has come much into vogue, and it is undoubtedly a valuable antiseptic. A disadvantage, however, to its use is that, when diluted and exposed to air, amongst other changes which it undergoes it may be transformed, under little understood conditions, into trioxymethylene and paraformaldehyde, these being polymers of formaldehyde. The bactericidal values of these mixtures are thus indefinite. Formalin may be used either by applying it in its liquid form or as a spray, or the gas which evaporates at ordinary temperatures from the solution may be utilised. To disinfect such an organic mixture as pus containing pyogenic organisms a 10 per cent solution acting for half an hour is necessary. In the case of pure cultures, a 5 per cent solution will kill the cholera organism in three minutes, anthrax bacilli in a quarter of an hour, and the spores in five hours. When such organisms as pyogenic cocci, cholera spirillum, and anthrax bacillus infect clothing, an exposure to the full strength of formalin for two hours is necessary, and in the case of anthrax spores, for twenty-four hours. Silk threads impregnated with the plague bacillus were found to be sterile after two minutes' exposure to formalin.

The action of formalin vapour has been much studied, as its use constitutes a cheap method of treating infected rooms, in which case some spray-producing machine is employed. It is stated that a mixture of 8 c.c. of formalin with 48 c.c. of water is sufficient when vapourised to disinfect one cubic metre, so far as non-sporing organisms are concerned. It is stated that 1 part

formalin in 10,000 of air will kill the cholera vibrio in one hour, diphtheria bacillus in three hours, the staphylococcus pyogenes in six hours, and anthrax spores in thirteen hours. In the case of organisms which have become dry it is probable, however, that much longer exposures are necessary, but on this point we have not definite information.

Formalin gas has only a limited application; it has little effect on dry organisms, and in the case of wet organisms, in order to be effective, probably must become dissolved so as to give the moisture a proportion analogous to the strengths stated above with regard to the vapour.

*Sulphurous Acid.*—This substance has long been in use, largely from the cheapness with which it can be produced by burning sulphur in the air. An atmosphere containing .98 per cent will kill the pyogenic cocci in two minutes if they are wet, and in twenty minutes if they are dry; and anthrax bacilli are killed by thirty minutes' exposure, but to kill anthrax spores an exposure of from one to two hours to an atmosphere containing 11 per cent is necessary. For a small room the burning of about a pound and a half (most easily accomplished by moistening the sulphur with methylated spirit) is usually considered sufficient. It has been found that if bacteria are protected, *e.g.* when they are in the middle of small bundles of clothes, no effect is produced even by an atmosphere containing a large proportion of the sulphurous acid gas. The practical applications of this agent are therefore limited.

*Potassium Permanganate.*—The action of this agent very much depends on whether it can obtain free access to the bacteria to be killed or whether these are present in a solution containing much organic matter. In the latter case the oxidation of the organic material throws so much of the salt out of action that there may be little left to attack the organisms. Koch found that to kill anthrax spores a 5 per cent solution required to act for about a day; for most organisms a similar solution acting for shorter periods has been found sufficient, and in the case of the pyogenic cocci a 1 per cent solution will kill in ten minutes. There is little doubt that such weaker solutions are of value in disinfecting the throat on account of their non-irritating properties, and good results in this connection have been obtained in cases of diphtheria. A solution of 1 in 10,000 has been found to kill plague bacilli in five minutes.

*Carbolic Acid.*—Of all the aromatic series this is the most extensively employed antiseptic. All ordinary bacteria in the vegetative condition, and of these the staphylococcus pyogenes

is the most resistant, are killed in less than five minutes by a 2-3 per cent solution in water, so that the 5 per cent solution usually employed in surgery leaves a margin of safety. But for the killing of such organisms as anthrax spores a very much longer exposure is necessary; thus Koch found it necessary to expose these spores for four days to ensure disinfection. The risk of such spores being present in ordinary surgical procedure may be overlooked, but there might be risk of tetanus spores not being killed, as these will withstand fifteen hours' exposure to a 5 per cent solution.

In the products of the distillation of coal there occur, besides carbolic acid, many bodies of a similar chemical constitution, and many mixtures of these are in the market—the chief being creolin, izal, and lysol, all of which are agents of value. Of these lysol is perhaps the most noticeable, as from its nature it acts as a soap and thus can remove fat and dirt from the hands. A one-third per cent solution is said to destroy the typhoid and cholera organisms in twenty minutes. A 1 per cent solution is sufficient for ordinary surgical procedures.

*Iodoform*.—This is an agent regarding the efficacy of which there has been much dispute. There is little doubt that it owes its efficiency to its capacity for being broken up by bacterial action in such a way as to set free iodine, which acts as a powerful disinfectant. The substance is therefore of value in the treatment of foul wounds, such as those of the mouth and rectum, where reducing bacteria are abundantly present. It acts more slightly where there are only pyogenic cocci, and it seems to have a specially beneficial effect in tubercular affections. In certain cases its action may apparently be aided by the presence of the products of tissue degeneration.

From the results which have been given it will easily be recognised that the choice of an antiseptic and the precise manner in which it is to be employed depend entirely on the environment of the bacteria which are to be killed. In many cases it will be quite impossible, without original inquiry, to say what course is likely to be attended with most success.

## CHAPTER V.

### RELATIONS OF BACTERIA TO DISEASE—THE PRODUCTION OF TOXINS BY BACTERIA.

**Introductory.**—It has already been stated that a strict division of micro-organisms into *saprophytes* and true *parasites* cannot be made. No doubt there are organisms such as the bacillus of leprosy which as yet have not been cultivated outside the animal body, and others, such as the gonococcus, which are in natural conditions always parasites associated with disease. But these latter can lead a saprophytic existence in specially prepared conditions, and there are many of the disease-producing organisms, such as the organisms of typhoid and cholera, which can flourish readily outside the body, even in ordinary conditions. The conditions of growth are, however, of very great importance in the study of the modes of infection in the various diseases, though they do not form the basis of a scientific division.

A similar statement applies to the terms *pathogenic* and *saprophytic*, and even to the terms *pathogenic* and *non-pathogenic*. By the term pathogenic is meant the power which an organism has of producing morbid changes or effects in the animal body, either under natural conditions or in conditions artificially arranged as in direct experiment. Now we know of no organisms which will in all circumstances produce disease in all animals, and, on the other hand, many bacteria described as harmless saprophytes will produce pathological changes if introduced in sufficient quantity. When, therefore, we speak of a pathogenic organism, the term is merely a relative one, and indicates that in certain circumstances the organism will produce disease, though in the science of human pathology it is often used for convenience as implying that the organism produces disease in man in *natural* conditions.

**Modifying Conditions.**—In studying the pathogenic effects in



any instance, both the micro-organisms and the animal affected must be considered, and not only the species of each, but also its exact condition at the time of infection. In other words, the resulting disease is the product of the sum total of the characters of the infecting agent, on the one hand, and of the subject of infection, on the other. We may, therefore, state some of the chief circumstances which modify each of these two factors involved and, consequently, the diseased condition produced.

1. *The Infecting Agent*.—In the case of a particular species of bacterium its effect will depend chiefly upon (a) its virulence, and (b) the number introduced into the body. To these may be added (c) the path of infection.

The *virulence*, *i.e.* the power of multiplying in the body and producing disease, varies greatly in different conditions, and the methods by which it can be diminished or increased will be afterwards described (*vide* Chapter XIX.). One important point is that when a bacterium has been enabled to invade and multiply in the tissues of an animal, its virulence for that species is often increased. This is well seen in the case of certain bacteria which are normally present on the skin or mucous surfaces. Thus it has been repeatedly proved that the bacillus coli cultivated from a septic peritonitis is much more virulent than that taken from the bowel of the same animal. The virulence may be still more increased by inoculating from one animal to another in series—the method of passage. Widely different effects are, of course, produced on the virulence being altered. For example, a streptococcus which produces merely a local inflammation or suppuration, may produce a rapidly fatal septicæmia when its virulence is raised. Virulence also has a relation to the animal employed, as occasionally on being increased for one species of animal it is diminished for another. For example, streptococci, on being inoculated in series through a number of mice, acquire increased virulence for these animals, but become less virulent for rabbits. (Knorr.) The theoretical consideration of virulence must be reserved for a later chapter (*see* Immunity).

The number of the organisms introduced, *i.e.* the dose of the infecting agent, is another point of importance. The healthy tissues can usually resist a certain number of pathogenic organisms of given virulence, and it is only in a few instances that one or two organisms introduced will produce a fatal disease, *e.g.* the case of anthrax in white mice. The healthy peritoneum of a rabbit can resist and destroy a considerable number of pyogenic micrococci without any serious result, but if a larger dose be

introduced, a fatal peritonitis may follow. Again, a certain quantity of a particular organism injected subcutaneously may produce only a local inflammatory change, but in the case of a larger dose the organisms may gain entrance to the blood stream and produce septicæmia. There is, therefore, for a particular animal, a minimum lethal dose which can be determined by experiment only; a dose, moreover, which is modified by various circumstances difficult to control.

*The path of infection*, may alter the result, serious effects often following a direct entrance into the blood stream. Staphylococci injected subcutaneously in a rabbit may produce only a local abscess, whilst on intravenous injection multiple abscesses in certain organs may result and death may follow. Local inflammatory reaction with subsequent destruction of the organisms may be restricted to the site of infection or may occur also in the lymphatic glands in relation. The latter therefore act as a second barrier of defence, or as a filtering mechanism which aids in protecting against blood infection. This is well illustrated in the case of "poisoned wounds." In some other cases, however, the organisms are very rapidly destroyed in the blood stream, and Klemperer has found that in the dog, subcutaneous injection of the pneumococcus produces death more readily than intravenous injection.

2. *The Subject of Infection*.—Amongst healthy individuals susceptibility and, in inverse ratio, resistance to a particular microbe may vary according to (a) species, (b) race and individual peculiarities, (c) age. Different species of the lower animals show the widest variation in this respect, some being extremely susceptible, others highly resistant. Then there are diseases, such as leprosy, gonorrhœa, etc., which appear to be peculiar to the human subject and have not yet been transmitted to animals. And further, there are others, such as cholera and typhoid, which do not naturally affect animals, and the typical lesions of which cannot be experimentally reproduced in them, or appear only imperfectly, although pathogenic effects follow inoculation with the organisms. In the case of the human subject, differences in susceptibility to a certain disease are found amongst different races and also amongst individuals of the same race, as is well seen in the case of tubercle and other diseases. Age also plays an important part, young subjects being more liable to certain diseases, *e.g.* to diphtheria. Further, at different periods of life certain parts of the body are more susceptible, for example, in early life, the bones and joints to tubercular and acute suppurative affections.

In increasing the susceptibility of a given individual, conditions of local or general diminished vitality play the most important part. It has been experimentally proved that conditions such as exposure to cold, fatigue, starvation, etc., all diminish the natural resistance to bacterial infection. Rats naturally immune can be rendered susceptible to glanders by being fed with phloridzin, which produces a sort of diabetes, a large amount of sugar being excreted in the urine (Leo). Guinea-pigs may resist subcutaneous injection of a certain dose of the typhoid bacillus, but if at the same time a sterilised culture of the bacillus coli be injected into the peritoneum, they quickly die of a general infection. Also a local susceptibility may be produced by injuring or diminishing the vitality of a part. If, for example, previous to an intravenous injection of staphylococci, the aortic cusps of a rabbit be injured, the organisms may settle there and set up an ulcerative endocarditis; or if a bone be injured, they may produce suppuration at the part, whereas in ordinary circumstances these lesions would not take place. The action of one species of bacterium is also often aided by the simultaneous presence of other species. In this case the latter may act simply as additional irritants which lessen the vitality of the tissues, but in some cases their presence also appears to favour the development of a higher degree of virulence of the former.

These facts, established by experiment (and many others might be given), illustrate the important part which local or general conditions of diminished vitality may play in the production of disease in the human subject. This has long been known by clinical observation. In normal conditions the blood and tissues of the body, with the exception of the skin and certain of the mucous surfaces, are bacterium-free, and if a few organisms gain entrance, they are destroyed. But if the vitality becomes lowered their entrance becomes easier and the possibility of their multiplying and producing disease greatly increased. In this way the favouring part played by fatigue, cold, etc., in the production of diseases of which the direct cause is a bacterium, may be understood. It is important to keep in view in this connection that many of the inflammation-producing and pyogenic organisms are normally present on the skin and various mucous surfaces. The action of a certain organism may devitalise the tissues to such an extent as to pave the way for the entrance of other bacteria; we may mention the liability of the occurrence of pneumonia, erysipelas, and various suppurative conditions in the course of or following infective fevers. In some cases the

specific organism may produce lesions through which the other organisms gain entrance, *e.g.* in typhoid, diphtheria, etc. A notable example of diminished resistance to bacterial infection is seen in the case of diabetes; tuberculosis and infection with pyogenic organisms are prone to occur in this disease and are of a severe character. It is not uncommon to find in the bodies of those who have died from chronic wasting disease, collections of micrococci or bacilli in the capillaries of various organs, which have entered in the later hours of life; that is to say, the bacterium-free condition of the blood has been lost in the period of prostration preceding death.

The methods by which the natural resistance may be specifically increased belong to the subject of immunity, and are described in the chapter on that subject.

**Modes of Bacterial Action.**—In the production of disease by micro-organisms there are two main factors involved, namely, (a) the multiplication of the living organisms after they have entered the body, and (b) the production by them of poisons which may act both upon the tissues around and upon the body generally. The former corresponds to infection, the latter is of the nature of intoxication or poisoning. In different diseases one of these is usually the more prominent feature, but both are always more or less concerned.

1. *Infection and Distribution of the Bacteria in the Body.*—After pathogenic bacteria have invaded the tissues, or in other words after infection by bacteria has taken place, their further behaviour varies greatly in different cases. In certain cases they may reach and multiply in the blood stream, producing a fatal septicæmia. In the lower animals this multiplication of the organisms in the blood throughout the body may be very extensive (for example, the septicæmia produced by the pneumococcus in rabbits); but in septicæmia in man, it very seldom, if ever, occurs to so great a degree, the organisms rarely remain in large numbers in the circulating blood, and their detection in it during life by microscopic examination is rare, and even culture methods may give negative results unless a large amount of blood is used. In such cases, however, the organisms may be found *post mortem* lying in large numbers within the capillaries of various organs, *e.g.* in cases of septicæmia produced by streptococci. In the human subject more frequently one of two things happens. In the first place, the organisms may remain local, producing little reaction around them, as in tetanus, or a well-marked lesion, as in diphtheria, pneumonia, etc. Or in the second place, they may pass by the lymph or blood stream to

other parts or organs in which they settle, multiply, and produce lesions, as in tubercle.

2. *Production of Chemical Poisons.*—In all these cases the growth of the organisms is accompanied by the formation of chemical products, which act generally or locally in varying degree as toxic substances. The toxic substances become diffused throughout the system, and their effects are manifested chiefly by symptoms such as the occurrence of fever, disturbances of the circulatory, respiratory, and nervous systems, etc. In some cases corresponding changes in the tissues are found, for example, the changes in the nervous system in diphtheria, to be afterwards described. The general toxic effects may be so slight as to be of no importance, as in the case of a local suppuration, or they may be very intense as in tetanus, or again, less severe but producing cachexia by their long continuance, as in tuberculosis.

The occurrence of local tissue changes or lesions produced in the neighbourhood of the bacteria, as already mentioned, is one of the most striking results of bacterial action, but these also must be traced to chemical substances formed in or around the bacteria, and either directly or through the medium of ferments. In this case it is more difficult to demonstrate the mode of action, for, in the tissues the chemical products are formed by the bacteria slowly, continuously, and in a certain degree of concentration, and these conditions cannot be exactly reproduced by experiment. It is also to be noted that more than one poison may be produced by a given bacterium, *e.g.* the tetanus bacillus (p. 380). Further, it is very doubtful whether all the chemical substances formed by a certain bacillus growing in the tissues are also formed by it in cultures outside the body. The separated toxin of diphtheria, like various vegetable and animal toxins (*vide infra*), however, possesses a local toxic action of very intense character, evidenced often by extensive necrotic change.

The injection of large quantities of many different pathogenic organisms in the *dead* conditions results in the production of a local inflammatory change which may be followed by suppuration, this effect being possibly brought about by certain substances in the bacterial protoplasm common to various species, or at least possessing a common physiological action (Buchner and others). When dead tubercle bacilli, however, are introduced into the blood stream, nodules do result in certain parts which have a resemblance to ordinary tubercles. In this case the bodies of the bacilli evidently contain a highly resistant and

slowly acting substance which gradually diffuses around and produces effects (*vide* Tuberculosis).

*Summary.*—We may say then that the action of bacteria as disease-producers, as in fact their power to exist and multiply in the living body, depends upon the chemical products formed directly or indirectly by them. This action is shown by *tissue changes* produced in the vicinity of the bacteria or throughout the system, and by *toxic symptoms* of great variety of degree and character.

We shall first consider the effects of bacteria on the body generally, and afterwards the nature of the chemical products.

### EFFECTS OF BACTERIAL ACTION.

These may be for convenience arranged in a tabular form as follows:—

#### A. *Tissue Changes.*

- (1) Local changes, *i.e.* changes produced in the neighbourhood of the bacteria.

Position (a) At primary lesion.

(b) At secondary foci.

Character (a) Tissue reactions } Acute or  
(b) Degeneration and necrosis } Chronic.

- (2) Produced at a distance from the bacteria, directly or indirectly, by the absorption of toxins.

(a) In special tissues—

(a) as the result of damage, *e.g.* nerve cells and fibres, secreting cells, vessel walls, or

(β) changes of a reactive nature in the blood-forming organs.

(b) General anatomical changes, the effects of malnutrition or of increased waste.

#### B. *Changes in Metabolism.*

The occurrence of fever, of errors of assimilation and elimination, etc.

**A. Tissue Changes produced by Bacteria.**—The effects of bacterial action are so various as to include almost all known pathological changes. However varied in character, they may be classified under two main headings:—(a) those of a degenerative or necrotic nature, the direct result of damage, and (b) those

of reactive nature, defensive or reparative. The former are the expression of the necessary vulnerability of the tissues, the latter of protective powers evolved for the benefit of the organism. In the means of defence both leucocytes and the fixed cells of the tissues are concerned. Both show phagocytic properties, *i.e.* have the power of taking up bacteria into their protoplasm. The cells are guided towards the focus of infection by chemiotaxis, and thus we find that different bacteria attract different cells. The most rapid and abundant supply of phagocytes is seen in the case of suppurative conditions where the neutrophile leucocytes of the blood are chiefly concerned. When the local lesion is of some extent there is usually an increase of these cells in the blood—a neutrophile leucocytosis. And further, recent observations have shown that associated with this there is in the bone-marrow an increased number of the mother-cells of these leucocytes—the neutrophile myelocytes. The passage of the neutrophile leucocytes from the marrow into the blood, with the resulting leucocytosis, is also apparently due to the absorbed bacterial toxins acting chemiotactically on the marrow. These facts abundantly show that the means of defence is not a mere local mechanism, but that increased proliferative activity in distant tissues is called into play. In addition to direct phagocytosis by these leucocytes, there is now abundant evidence that an important function is the production in the body of bactericidal and other antagonistic substances. In other cases the cells chiefly involved are the mononuclear hyaline leucocytes, and with them the endothelial cells, *e.g.* of serous membranes, often play an important part in the defence; this is well seen in typhoid fever, where the specific bacillus appears to have little or no action on the neutrophile leucocytes. In other cases, again, the reaction is chiefly on the part of the connective cells, though their proliferation is always associated with some variety of leucocytic infiltration and usually also with the formation of new blood vessels. Such a connective tissue reaction occurs especially in slow infections or in the later stages of an acute infection. The tissue changes resulting from cellular activity in the presence of bacterial invasion are naturally very varied—examples of this will be found in subsequent chapters—but they may be said to be manifestations of the two fundamental processes of (a) increased functional activity—movement, phagocytosis, secretion, etc.—and (b) increased formative activity—cell growth and division. The exudation from the blood vessels has been variously interpreted. There is no doubt that the exudate has bactericidal properties and also acts as a diluting

agent, but it must still be held as uncertain whether the process of exudation ought to be regarded as primarily defensive or as the direct result of damage to the endothelium of the vessels. It may also be pointed out that the various changes referred to are none of them peculiar to bacterial invasion; they are examples of the general laws of tissue change under abnormal conditions, and they can all be reproduced by chemical substances in solution or in a particulate state. What constitutes their special feature is their progressive or spreading nature, due to the bacterial multiplication.

(1) *Local Lesions*.—In some diseases the lesion has a special site; for example, the lesion of typhoid fever and, to a less extent, that of diphtheria. In other cases it depends entirely upon the point of entrance, *e.g.* malignant pustule and the conditions known as wound infections. In others again, there is a special tendency for certain parts to be affected, as the upper parts of the lungs in tubercle. In some cases the site has a mechanical explanation.

When organisms gain an entrance to the blood from a primary lesion, the organs specially liable to be affected vary greatly in different diseases. Pyogenic cocci show a special tendency to settle in the capillaries of the kidneys and produce miliary abscesses, whilst these lesions rarely occur in the spleen. On the other hand, the nodules in disseminated tubercle or glands are much more numerous in the spleen than in the kidneys, which in the latter disease are usually free from them. The important point is that the position of the disseminated lesions is not to be explained by a mechanical process, such as embolism, but depends upon a special relation between the organisms and the tissues, which may be spoken of either as a selective power on the part of the organisms or a special susceptibility of tissues, possibly in part due to their affording to the organisms more suitable conditions of nutriment. Even in the case of the lesions produced by dead tubercle bacilli, a certain selective character is observed.

*Acute Local Lesions*.—The local inflammatory reaction presents different characters in different conditions. It may be accompanied by abundant fibrinous exudation, or by great catarrh (in the case of an epithelial surface), or by hæmorrhage, or by oedema; it may be localised or spreading in character; it may be followed by suppuration, and may be accompanied or lead up to necrosis of the tissues of the part, a good example of the latter event being found in a boil. Examples will be given in subsequent chapters. The necrotic or degenerative changes



affecting especially the more highly developed elements of tissues are chiefly produced by the direct action of the bacterial poisons, though aided by the disturbances of nutrition involved in the vascular phenomena. It may here be pointed out that a well-marked inflammatory reaction is often found in animals which occupy a medium position in the scale of susceptibility, and that an organism which causes a general infection in a certain animal may produce only a local inflammation when its virulence is lessened.

*Chronic Local Lesions.*—In a considerable number of diseases produced by bacteria the local tissue reaction is a more chronic process than those described. In other words, the specific irritant is less intense, so that there is less vascular disturbance and a greater preponderance of the proliferative processes, leading to new formation of connective tissue or a modified connective tissue. This formation may occur in foci here and there, so that nodules of greater or less consistence result, or it may be more diffuse. Such changes especially occur in the diseases often known as the *infective granulomata*, of which tubercle, leprosy, glanders, actinomycosis, syphilis, etc., are examples. A hard and fast line, however, cannot be drawn between such conditions and those described above as acute. In glanders, for example, especially in the human subject, the lesion often approaches very nearly to an acute suppurative change, and sometimes actually is of this nature. Whilst in these diseases the fundamental change is the same—viz. a reaction to an irritant of minor intensity—the exact structural characters and arrangement vary in different diseases. In some cases the disease may be identified by the histological changes alone, but on the other hand, this is often impossible. These changes often include the occurrence of degenerations or of actual necrosis in the newly formed tissue. In the granulomata, infection of other parts from the primary lesion takes place chiefly by the blood vessels and lymphatics, though sometimes along natural tubes such as the bronchi, intestine, etc.

(2) *General Lesions produced by Toxins.*—In the various infective conditions produced by bacteria, changes commonly occur in certain organs unassociated with the presence of the bacteria; these are produced by the action of bacterial products circulating in the blood. Many such lesions can be produced experimentally. The secreting cells of various organs, especially the kidney and liver, are specially liable to change of this kind. Cloudy swelling, which may be followed by fatty change or by actual necrosis with granular disintegration, is

common. Hyaline change in the walls of arterioles may occur, and in certain chronic conditions waxy change is brought about in a similar manner. The latter has been produced in animals by the repeated injection of the *staphylococcus aureus*. Capillary hæmorrhages are not uncommon, and are in many cases due to an increased permeability of the vessel walls, aided by changes in the blood plasma, as evidenced sometimes by diminished coagulability. Similar hæmorrhages may follow the injection of some bacterial toxins, *e.g.* of diphtheria, and also of vegetable poisons, *e.g.* ricin and abrin. Skin eruptions occurring in the exanthemata are probably produced in the same way, though in many of these diseases the causal organism has not yet been isolated. We have, however, the important fact that corresponding skin eruptions may be produced by poisoning with certain drugs. In the nervous system degenerative changes have been found in diphtheria, both in the spinal cord and in the peripheral nerves, and have been reproduced experimentally by the products of the diphtheria bacilli. There is also experimental evidence that the *bacillus coli communis* and the *streptococcus pyogenes* may, by means of their products, produce areas of softening in the spinal cord, and this may furnish an explanation of some of the lesions found clinically. It is also possible that some serous inflammations may be produced in the same way.

**B. Disturbances of Metabolism, etc.**—It will easily be realised that such profound tissue changes as have been detailed cannot occur without great interference with the normal bodily metabolism. General malnutrition and cachexia are of common occurrence, and it is a striking fact found by experiment that after injection of bacterial products, *e.g.* of the diphtheria bacillus, a marked loss of body weight often occurs which may be progressive, leading to the death of the animal. In bacterial disease assimilation is often imperfect, for the digestive glands are affected, it may be, by actual poisoning by bacterial products, it may be by the occurrence of fever. The fatty degenerations which are so common are indicative of a breaking down of the proteid molecules, and are associated with increased urea production, while the degeneration of the kidney epithelium renders the excretion of waste products deficient or impossible, and this is not infrequently the immediate cause of death. But of all the changes in metabolism the most difficult to understand is the occurrence of that interference with the heat-regulating mechanism which results in fever. The degree and course of the latter vary, sometimes conforming to a more or less definite type,

~~where the bacilli are~~ selective in their field of operation, as in croupous pneumonia or typhoid, sometimes being of a very irregular kind, especially when the bacteria from time to time invade fresh areas of the body, as in pyæmic affections. The main point of interest regarding the development of fever is as to whether it is a direct effect of the circulation of bacterial toxins, or if it is to be looked on as part of the reaction of the body against the irritant. This question has still to be settled, and all that we can do is to adduce certain facts bearing on it. Thus in diphtheria and tetanus, where toxic action leading to degeneration plays such an important part, fever may be a very subsidiary feature, except in the terminal stage of the latter disease; and in fact in diphtheria profoundly toxic effects may be produced with little or no interference with heat regulation. On the other hand, in bacterial disease, where defensive and reparative processes predominate, fever is rarely absent, and it is nearly always present when an active leucocytosis is going on. In this connection it may be remarked that several observers have found that, when a relatively small amount of the dead bodies of certain bacteria are injected into an animal, fever occurs; while the injection of a large amount of the same is followed by subnormal temperatures and rapidly fatal collapse. It might appear as if this indicated that the occurrence of fever had a beneficial effect, but this is one of the points at issue. Certainly such an effect is not due to the bacteria being unable to multiply at the higher degrees of temperature occurring in fever, for this has been shown not to be the case. Whether the increase of bodily temperature indicates the occurrence of changes resulting in the production of bactericidal bodies, etc., is very doubtful; a production of antagonistic substances may be effected without the occurrence of fever or of any apparent disturbance of health. If we consider the site of the heat production in fever we again are in difficulties. It might appear as if the tissue destruction, indicated by the occurrence of fatty degeneration, would lead to heat development, but frequently excessive heat production with increased proteid metabolism occurs without any discoverable changes in the tissues; and further, in phosphorus poisoning there is little fever with great tissue destruction. The increased work performed by the heart in most bacterial infections no doubt contributes to the rise of bodily temperature. But we must bear in mind that in fever there is more than mere increase of heat production—there is also a diminished loss of heat from interference with the nervous mechanism of the sweat apparatus. The known facts would

indicate that in fever there is a factor involving the nervous system to be taken into account. The whole subject is thus very obscure.

*Symptoms.*—Many of the symptoms occurring in bacterial affections are produced by the histological changes mentioned, as can be readily understood; whilst in the case of others, corresponding changes have not yet been discovered. Of the latter those associated with fever, with its disturbances of metabolism and manifold affections of the various systems, are the most important. The nervous system is especially liable to be affected—convulsions, spasms, coma, paralysis, etc., being common. The symptoms due to disturbance or abolition of the functions of secretory glands also constitute an important group, forming, as they do, a striking analogy to what is found in the action of various drugs.

These tissue changes and symptoms are given only as illustrative examples, and the list might easily be greatly amplified. The important fact, however, is that *nearly all, if not quite all, the changes found throughout the organs (without the actual presence of bacteria), and also the symptoms occurring in infective diseases, can either be experimentally reproduced by the injection of bacterial poisons or have an analogy in the action of drugs.*

#### THE TOXINS PRODUCED BY BACTERIA.

**Early Work on Toxins.**—We know that bacteria are capable of giving rise to poisonous bodies within the animal body and also in artificial media. We know, however, comparatively little of the actual nature of such bodies, and therefore we apply to them as a class the general term *toxins*. The necessity for accounting for the general pathogenic effects of certain bacteria, which in the corresponding diseases were not distributed throughout the body, directed attention to the probable existence of such toxins; and the first to systematically study the production of such poisonous bodies was Brieger. This observer isolated from putrefying substances, and also from bacterial cultures, nitrogen-containing bodies, which he called *ptomaines*. Similar bodies occurring in the ordinary metabolic processes of the body had previously been described and called *leucomaines*. Ptomaines isolated from pathogenic bacteria in no case reproduced the symptoms of the disease, except perhaps in tetanus and this only owing to their impurity. The methods by which they were isolated were faulty, and they have therefore only a historic interest.

The introduction of the principle of rendering fluid cultures bacteria-free by filtration through unglazed porcelain, and its application by Roux and Yersin to obtain, in the case of the *b. diphtheriae*, a solution containing a toxin which reproduced the symptoms of this disease (*vide* Chap. XV.), encouraged the further inquiry as to the nature of this toxin. An attempt on the part of Brieger and Fraenkel to obtain a purified diphtheria toxin by precipitating bouillon cultures by alcohol (the product being denominated a toxalbumin) did not greatly advance knowledge on the subject, and further investigation soon showed that characteristic toxins can be isolated from but few bacteria.

**General Facts regarding Bacterial Toxins.**—The following may be regarded as the chief facts regarding bacterial toxins which have been revealed by the study, partly of the bodily tissues of animals infected by the bacteria concerned, partly of artificial cultures of these bacteria. The dead bodies of certain bacteria have been found to be very toxic. When, for instance, tubercle bacilli are killed by heat and injected into the body tissues of a susceptible animal tubercular nodules are found to develop round the sites where they have lodged. From this it is inferred that they must have contained characteristic toxins, seeing that characteristic lesions result. The bodies of the cholera vibrio are likewise toxic. Such *intracellular* toxins, as they have been called, may appear in the fluids in which the bacteria are living (1) by excretion in an unaltered or altered condition, (2) by the disintegration of the bodies of the organisms which we know are always dying in any bacterial growth. The death of bacteria occurs also in the body of an infected animal, and the disintegration of these dead bacteria constitutes an important means by which the poisons they contain are absorbed. There is some evidence that often bacteria produce during growth poisons which are hurtful to their own vitality, and also that ferments are produced by them which have a solvent effect on the poisoned members of the colony. Such a process of *autolysis*, as it has been called, may have an important effect in liberating intracellular toxins. We do not, however, understand all that takes place under such circumstances; for the dead bodies of many bacteria, such as those of anthrax and diphtheria, are relatively non-toxic. As it is impossible, at present, to obtain intracellular toxins apart from other derivatives of the bacterial protoplasm, all our knowledge concerning their effects is derived from the study of what happens when the bodies of bacteria killed by chloroform vapour or by heat are

injected into animals. When effects are produced by such injections they do not present in any particular case specific characters. They are of the nature of general disturbances of metabolism, as manifested by fever, loss of weight, etc., often of such serious degree as to result in death. It is important to note that when pathogenic effects are produced these usually appear very soon, it may be in a few hours after injection of the toxic material; there is not the definite period of incubation which with other toxins often elapses before symptoms appear.

Sometimes the media in which bacteria are growing become extremely toxic. This is more marked in some cases than in others. The two best examples of bacteria thus producing soluble toxins are the diphtheria and tetanus bacilli. In these and similar cases when bouillon cultures are filtered bacterium-free by means of a porcelain filter, toxic fluids are obtained, which on injection into animals reproduce the highly characteristic symptoms of the corresponding diseases. In the case of the *b. anthracis* and of many others, at any rate when growing in artificial media, such toxin production is much less marked, a filtered bouillon culture being relatively non-toxic. Poisons appearing in culture media have been called *extra-cellular* toxins, but we cannot as yet say whether they are excreted by the bacteria or whether they are produced by the bacteria acting on the constituents of the media. We therefore cannot as yet draw a hard and fast line between intra- and extracellular toxins, but the terms are convenient, and may apply to two actually different sets of bodies. That the poisonous capacities of a bacterium may be very complicated is shown by what is known in the case of the cholera vibrio, where the poisons which dissolve out into the culture fluid are probably different in their nature from those which act when the dead bacteria are injected into an animal. The extracellular toxins are the more easily obtainable in large quantities, and it is their nature and effects which are best known. No method, however, has been discovered of obtaining them in a pure form, and our knowledge of their properties is exclusively derived from the study of the toxic filtrates of bouillon cultures—these filtrates being usually referred to simply as the toxins. These toxins differ in their effects from the intracellular poisons in that specific actions on certain tissues are often manifested. Thus the toxins of the diphtheria, the tetanus, and the botulism bacilli all act on the nervous system; with some of the pyogenic bacteria, on the other hand, poisons, probably of similar nature, produce solution of red blood corpuscles (this

last may explain, in part at least, the anæmias so common in the associated diseases). In the action of many of these toxins the occurrence of a period of incubation between the introduction of the poison into the animal tissues and the appearance of symptoms is often a feature.

We have seen that in certain cases there is difficulty in understanding the action of bacteria which do not form toxins in fluid media, especially as in the cases of some of these the bacterial protoplasm does not seem very toxic. Yet we often see effects produced at a distance from the focus of infection, *e.g.* in anthrax. To explain such occurrences it has long been put forward as a possibility that some bacteria are only capable of producing toxins within the animal tissues, and it has further been thought possible that bacteria, such as, for example, the typhoid bacillus, which do in media give rise to intracellular toxins, might either produce these toxins more readily in the tissues or might produce in addition other toxins of a different nature. Recently such toxins have been much studied, and the name *aggressins* has been given to them. The evidence adduced for the existence of these aggressins as a separate group of bacterial poisons is of the following kind. An animal is killed by a dose of the typhoid, dysentery, cholera, or tubercle bacillus, or by a staphylococcus, the organism being introduced into one of the serous cavities. After death the serous exudation, which in all these cases is present, is removed, and centrifugalised to remove the bacteria so far as this can be done by such a procedure; the bacteria which are left are killed by shaking the fluid up with toluol and leaving it to stand for some days. It is stated that such a fluid is of itself without pathogenic effect, but has the property of transforming a non-lethal dose of the bacterium used into one having fatal effect. Further, the effects of the combined actions of the bacteria and aggressins are often of a much more acute character than can be obtained with toxic products developed *in vitro*. Thus, in the case of the action of a non-lethal dose of the tubercle bacillus plus its aggressin, death may occur in twenty hours, a result never obtained with artificial cultures of the organism. The results obtained are attributed to a paralysing action which the aggressin is supposed to have on the phagocytic functions of the leucocytes. The subject is full of difficulties, and in the case of certain of the organisms employed, it is stated that results similar to those attributed to aggressin action have been observed with macerated cultures,—the deduction being that in the aggressins we are merely dealing with concentrated intracellular toxins. On the other hand,

as evidence of the existence of a special group of toxins, it has been stated that a special type of immunity against the aggressins can be originated. Perhaps the most important aspect of the controversy is the recognition of the existence of toxins having an action on the leucocytes. A poison causing death of these cells in connection with the pus-forming action of the pyogenic cocci has been described under the name of leucocidin. The investigation of such poisons must be of the highest importance in view of the part played by the blood-cells in the protection of the body against infection, and it is possible that toxins having a fatal effect in strong concentrations, may, when dilute, be responsible for the phenomena of attraction or repulsion of leucocytes which we know occur round a focus of bacterial growth in the body.

It is to be noted that in the case of any particular bacterium several different toxins may be at work, and it is also possible that one toxin may have different effects on different tissues of the body. Intracellular toxins of an organism may cause general metabolic disturbances, and its special toxins may act on special tissues. Thus the *staphylococcus pyogenes aureus* may cause fever, wasting, etc., by its intracellular poisons, a special action on the leucocytes by a leucocidin toxin, and anaemia by its haemolytic properties. The phenomena of any bacterial disease may thus in reality be due to very different and complex causes.

**The Nature of Toxins.**—There is still comparatively little known regarding this subject, and it chiefly relates to the extracellular toxins. The earlier investigations upon toxins suggested that analogies exist between the modes of bacterial action and what takes place in ordinary gastric digestion, and the idea was worked out for anthrax, diphtheria, tetanus, and ulcerative endocarditis by Sidney Martin. This observer took, not solutions artificially made up with albumoses,<sup>1</sup> but the natural fluids of the body or definite

<sup>1</sup> In the digestion of albumins by the gastric and pancreatic juices the albumoses are a group of bodies formed preliminarily to the production of peptone. Like the latter they differ from the albumins in their not being coagulated by heat, and in being slightly dialysable. They differ from the peptones in being precipitated by dilute acetic acid in presence of much sodium chloride, and also by neutral saturated sulphate of ammonia. Both are precipitated by alcohol. The first albumoses formed in digestion are proto-albumose and hetero-albumose, which differ in the insolubility of the latter in hot and cold water (insolubility and coagulability are quite different properties). They have been called the primary albumoses. By further digestion both pass into the secondary albumose, deuterio-albumose, which differs slightly in chemical reactions from the parent bodies, *e.g.* it cannot be precipitated from watery solutions by saturated sodium chloride unless a



solutions of albumins, and, further, never subjected the results of the bacterial growth to heat above 40° C., or to any stronger agent than absolute alcohol. He found that albumoses and sometimes peptones were formed by the action of the pathogenic bacteria studied, and further, that the precipitate containing these albumoses was toxic. In certain cases the process of splitting up of the albumins went further than in peptic digestion, and organic bases or acids might be formed. According to Martin, the characteristic symptoms of the diseases could be explained by compound actions, in which the albumoses were responsible for some of the effects, the remaining bodies for others. A similar digestive action has been traced in the case of the tubercle bacillus by Kühne.

Further evidence that bacterial toxins are either albumoses or bodies having a still smaller molecule is furnished by C. J. Martin. This worker, by filling the pores of a Chamberland bougie with gelatin, has obtained what is practically a strongly supported colloid membrane through which dialysis can be made to take place under great pressure, say, of compressed oxygen. He finds that in such an apparatus toxins,—at least two kinds tried,—will pass through just as an albumose will.

Brieger and Boer, working with bouillon cultures of diphtheria and tetanus, have, by precipitation with zinc chloride, separated bodies which show characteristic toxic properties, but which have the reactions neither of peptone, albumose, nor albuminate, and the nature of which is unknown. It has also been found that the bacteria of tubercle, tetanus, diphtheria, and cholera can produce toxins when growing in proteid-free fluids. In the case of diphtheria when the toxin is produced in such a fluid a proteid reaction appears. Of course this need not necessarily be caused by the toxin. Further investigation is here required, for Uschinsky, applying Brieger and Boer's method to a toxin so produced, states that the toxic body is not precipitated by zinc salts, but remains free in the medium. If the toxins are really non-proteid they may, on the one hand, be the final product of a digestive action, or they may be the manifestation of a separate vital activity on the part of the bacteria. On the latter theory the toxicity of the toxic albumoses of Sidney Martin may be due to the precipitation of the true toxins along with these other bodies. From the chemical standpoint this is quite possible. When we take into account the extraordinary potency of these poisons (in

trace of acetic acid be present. Dysalbumose is probably merely a temporary modification of hetero-albumose. Further digestion of deuterio-albumose results in the formation of peptone.

the case of tetanus the fatal dose of the pure poison for a guinea-pig must often be less than  $\cdot 000001$  gr.), we can understand how attempts by present chemical methods to isolate them in a pure condition are not likely to be successful, and of their real nature we know nothing. In a recent research Friedberger and Moreschi have shown that the intravenous injection in the human subject of a fraction of a loopful of a dead typhoid culture gives rise to toxic symptoms, including marked febrile reaction. Such injections are followed by the appearance of agglutinating and bacteriolytic substances in the serum. These results show that intracellular toxins may be comparable with extra-cellular toxins so far as concerns the extremely small dose sufficient to produce toxic effects.

Amongst the properties of the extracellular toxins are the following. They are certainly all uncrystallisable; they are soluble in water and they are dialysable; they are precipitated along with proteids by concentrated alcohol, and also by ammonium sulphate; if they are proteids they are either albumoses or allied to the albumoses; they are often relatively unstable, having their toxicity diminished or destroyed by heat (the degree of heat which is destructive varies much in different cases), light, and by certain chemical agents. Their potency is often altered in the precipitations practised to obtain them in a pure or concentrated condition, but among the precipitants ammonium sulphate has little if any harmful effect. Regarding the toxins which are more intimately associated with the bacterial protoplasm we know much less, but it is probable that their nature is similar, though some of them at least are not so easily injured by heat, *e.g.* those of the tubercle bacillus, already mentioned. In the case of all toxins the fatal dose for an animal varies with the species, body weight, age, and previous conditions as to food, temperature, etc. In estimating the minimal lethal dose of a toxin these factors must be carefully considered.

The following is the best method of obtaining concentrated extracellular toxins. The toxic fluid is placed in a shallow dish, and ammonium sulphate crystals are well stirred in till no more dissolve. Fresh crystals to form a bulk nearly equal to that of the whole fluid are added, and the dish set in an incubator at  $37^{\circ}$  C. over night. Next day a brown scum of precipitate will be found floating on the surface. This contains the toxin. It is skimmed off with a spoon, placed in watch glasses, and these are dried *in vacuo* and stored in the dark, also *in vacuo*, or in an exsiccator containing strong sulphuric acid. For use the contents of one are dissolved up in a little normal saline solution.

The comparison of the action of bacteria in the tissues in the production of these toxins to what takes place in the gastric

digestion, has raised the question of the possibility of the elaboration by these bacteria of *ferments* by which the process may be started. Thus Sidney Martin puts forward the view that ferments may be produced, which we may look on as the primary toxic agents, and which act by digesting surrounding material and producing albumoses,—these poisons being, as it were, secondary poisons. Hitherto all attempts at the isolation of bacterial ferments of such a nature have failed.

But apart from the fact that with such bacteria as these of tetanus and diphtheria, a digestive action may occur, analogies have been drawn between ferment and toxic action. The chief facts upon which such analogies have been founded are as follows. Thus the toxic products of these and other bacteria lose their toxicity by exposure to a temperature which puts an end to the activity of such an undoubted ferment as that of the gastric juice. If a bouillon containing diphtheria toxin be heated at 65° C. for one hour, it is found to have lost much of its toxic effect, and in the case of *b. tetani* all the toxicity is lost by exposure at this temperature. In both diseases there is a still further fact which is adduced in favour of the toxic substances being of the nature of ferments, namely, the existence of a definite period of incubation between the injection of the toxic bodies and the appearance of symptoms. This may be interpreted as showing that after the introduction of, say, a filtered bouillon culture, further chemical substances are formed in the body before the actual toxic effect is produced. Too much reliance must not be placed on such an argument, for in the case of tetanus, at least, the delay may be explained by the fact that the poison apparently has to travel up the nerve trunks before the real poisonous action is developed. Further, with some poisons presently to be mentioned which are closely allied to the bacterial toxins an incubation period may not exist. It would not be prudent to dogmatise as to whether the toxins do or do not belong to such an ill-defined group of substances as the ferments. It may be pointed out, however, that the essential concept of a ferment is that of a body which can originate change without itself being changed, and no evidence has been adduced that toxins fulfil this condition. Another property of ferments is that so long as the products of fermentation are removed, the action of a given amount of ferment is indefinite. Again, in the case of toxins no evidence of such an occurrence has been found. A certain amount of a toxin is always associated with a given amount of disease effect, though a process of elimination of waste products must be all the time

going on in the animal's body. Again, too much importance must not be attached to loss of toxicity by toxins at relatively low temperatures. This is not true of all toxins, and furthermore many proteids show a tendency to change at such temperatures; for instance, if egg albumin be kept long enough at 55° C. nearly the whole of it will be coagulated. We must therefore maintain an open mind on this subject.

**Similar Vegetable and Animal Poisons.**—Within recent years it has been found that the bacterial poisons belong to a group of toxic bodies all presenting very similar properties, other members of which occur widely in the vegetable and animal kingdoms. Among plants the best-known examples are the ricin and abrin poisons obtained by making watery emulsions of the seeds of the *Ricinus communis* and the *Abrus precatorius* (jequirity) respectively. From the *Robinia pseudacacia* another poison—robin—belonging to the same group is obtained. The chemical reactions of ricin and abrin correspond to those of the bacterial toxins. They are soluble in water, they are precipitable by alcohol, but being less easily dialysable than the albumoses they have been called toxalbumins. Their toxicity is seriously impaired by boiling, and they also gradually become less toxic on being kept. Both are among the most active poisons known—ricin being the more powerful. When they are injected subcutaneously a period of twenty-four hours usually elapses—whatever be the dose—before symptoms set in. Both tend to produce great inflammation at the seat of inoculation, which in the case of ricin may end in an acute necrosis; in fatal cases hæmorrhagic enteritis and nephritis may be found. Both act as irritants to mucous membranes, abrin especially being capable of setting up most acute conjunctivitis.

It is also certain that the poisons of scorpions and of poisonous snakes belong to the same group. The poisons derived from the latter are usually called venins, and a very representative group of such venins derived from different species has been studied. To speak generally there is derivable from the natural secretions of the poison glands a series of venins which have all the reactions of the bodies previously considered. Like ricin and abrin, they are not so easily dialysable as bacterial toxins, and therefore have also been classed as toxalbumins. Their properties are also similar; many of them are destroyed by heat, but the degree necessary here also varies much, and some will stand boiling. There is also evidence that in a crude venin there may be several poisons differently sensitive to heat. All the venins are very powerful poisons, but here there is practically no period of incubation—the effects are almost immediate. An outstanding feature of the venins is the complexity of the crude poison secreted by any particular species of snake. C. J. Martin in summing up the results of many observers has pointed out that different venoms have been found to contain one or more of the following poisons: a neurotoxin acting on the respiratory centre, a neurotoxin acting on the nerve-endings in muscle, a toxin causing hæmolysis, toxins acting on other cells, e.g. the endothelium of blood-vessels (this from its effects has been named hæmorrhagin), leucocytes, nerve-cells, a toxin causing thrombosis, a toxin having an opposite effect and preventing coagulation, a toxin neutralising the bactericidal qualities of the body fluids and thus favouring putrefaction, a toxin causing agglutination of the red blood corpuscles, a proteolytic

ferment, a toxin causing systolic standstill of the excised heart. Any particular venom contains a mixture in varying proportions of such toxins, and the different effects produced by the bites of different snakes largely depend on this variability of composition. The neurotoxic, the thrombotic, and the hamolytic toxins are very important constituents of any venom. The toxicity of different venoms varies much, and no general statement can be made with regard to the toxicity of different poisons towards man. Lamb has calculated that the fatal dose of crude cobra venom for man is probably about .015 of a gramme, and that if such a snake bites with full glands many times this dose would probably be injected, but, of course, the amount emitted depends largely on the period which has elapsed since the animal last emptied its glands. When a dose of a venom not sufficient to cause immediate death from general effects be given, very rapid and widespread necrosis often may occur in a few hours round the site of inoculation.

An extremely important fact was discovered by Flexner and Noguchi, namely, that the hamolytic toxin of cobra venom in certain cases has no action by itself, but produces rapid solution of red corpuscles when some normal serum is added, the latter containing a labile complement-like body, which activates the venom. In this there is a close analogy to what holds in the case of a hamolytic serum deprived of complement by heat at 55° C. (p. 479). Kyes and Sachs further showed that in addition to serum-complement a substance with definitely known constitution, namely lecithin, had the property of activating the hamolytic substance in cobra venom, the two apparently uniting to form an actively toxic substance. Later still, Kyes succeeded in demonstrating the union of the two substances to form a cobra-lecithid, and in separating the latter as a practically pure compound, which is, unlike lecithin, insoluble in ether, but soluble in chloroform. So far no example of activating a bacterial toxin is known, but the results mentioned point to the possibility of this occurring in some cases in the tissues of the body.

**The Theory of Toxic Action.**—While we know little of the chemical nature of any toxins we may, from our knowledge of their properties, group together the tetanus and diphtheria poisons, ricin, abrin, snake poisons, and scorpion poisons. Besides the points of agreement already noted, all possess the further property that, as will be afterwards described, when introduced into the bodies of susceptible animals they stimulate the production of substances called antitoxins. The nature of the antagonism between toxin and antitoxin will be discussed later. Here, to explain what follows it may be stated (1) that the molecule of toxin most probably forms a chemical combination with the molecule of antitoxin, and (2) that it has been shown that toxin molecules may lose much of their toxic power and still be capable of uniting with exactly the same proportion of antitoxin molecules. From these and other circumstances Ehrlich has advanced the view that the toxin molecule has a very complicated structure, and contains two atom groups. One of these, the *haptophorous* (ἅπτειν, to bind to), is that by which com-

ination takes place with the antitoxin molecule, and also with presumably corresponding molecules naturally existing in the tissues. The other atom group he calls the *toxophorous*, and it is to this that the toxic effects are due. This atom group is bound to the cell elements, *e.g.* the nerve cells in tetanus, by the *haptophorous* group. Ehrlich explains the loss of toxicity which with time occurs in, say, diphtheria toxin, on the theory that the *toxophorous* group undergoes disintegration. And if we suppose that the *haptophorous* group remains unaffected we can then understand how a toxin may have its toxicity diminished and still require the same proportion of antitoxin molecules for its neutralisation. To the bodies whose *toxophorous* atom groups have become degenerated, Ehrlich gives the name *toxoids*. The theory may afford an explanation of what has been suspected, namely, that in some instances toxins derived from different sources may be related to one another. For example, Ehrlich has pointed out that ricin produces in a susceptible animal body an antitoxin which corresponds almost completely with that produced by another vegetable poison, robin (*vide supra*), though ricin and robin are certainly different. This may be explained according to the view that robin is a *toxoid* of ricin, *i.e.* their *haptophorous* groups correspond, while their *toxophorous* differ. The evidence on which Ehrlich's deductions are based is of a very weighty character, and will be again referred to in the chapter on Immunity.

With regard to the intracellular toxins we shall see it is difficult to determine whether or not they share with the extracellular poisons the property of stimulating antitoxin formation, —if they do not, then they may belong to an entirely different class of substances. It is certain that a tolerance against such poisons is difficult to establish and is not of a lasting character. We thus cannot say what the mechanism is by which these poisons act. It may be said that Macfadyen by grinding up typhoid bacilli frozen by liquid air claimed that on thawing he obtained the intracellular toxins in liquid form, and he further stated that by using this fluid he could immunise animals not only against the toxins but also against the living bacteria.

We have already pointed out that those who claim for the *aggressins* a special character hold that the activity of these bodies has as its effect the interference with the phagocytic functions of the leucocytes. They also hold that a special type of immunity can be developed against the aggressive bodies.

## CHAPTER VI.

### INFLAMMATORY AND SUPPURATIVE CONDITIONS.

THIS subject is an exceedingly wide one, and embraces a great many pathological conditions which in their general characters and results are widely different. Thus in addition to suppuration, various inflammations, ulcerative endocarditis, septicæmia and pyæmia, will come up for consideration. With regard to these the two following general statements, established by bacteriological research, may be made in introducing the subject. In the first place, there is no one specific organism for any one of these conditions; various organisms may produce them, and not infrequently more than one organism may be present together. In the second place, the same organism may produce widely varying results under different circumstances,—at one time a local inflammation or abscess, at another multiple suppurations or a general septicæmia. The principles on which this diversity in results depends have already been explained (p. 151). Furthermore, there are conditions like acute pneumonia, epidemic meningitis, acute rheumatism, etc., which have practically the character of specific diseases and yet which as regards their essential pathology belong to the same class. The arrangement followed is to a certain extent one of convenience.

It may be well to emphasise some of the chief points in the pathology of these conditions. In *suppuration* the two main phenomena are—(a) a progressive immigration of leucocytes, chiefly of the polymorpho-nuclear (neutrophile) variety, and (b) a liquefaction or digestion of the supporting elements of the tissue along with necrosis of the cells of the part. The result is that the tissue affected becomes replaced by the cream-like fluid called pus. A suppurative inflammation is thus to be distinguished on the one hand from an inflammation without destruction of tissue, and on the other from necrosis or death

*en masse*, where the tissue is not liquefied, and leucocyte accumulation may be slight. When, however, suppuration is taking place in a very dense fibrous tissue, liquefaction may be incomplete, and a portion of dead tissue or slough may remain in the centre, as is the case in boils. In the case of suppuration in a serous cavity the two chief factors are the progressive leucocytic accumulation and the disappearance of any fibrin which may be present.

Many experiments have been performed to determine whether suppuration can be produced in the absence of micro-organisms by various chemical substances, such as croton oil, nitrate of silver, turpentine, etc.—care, of course, being taken to ensure the absence of bacteria. The general result obtained by independent observers is that as a rule suppuration does not follow, but that in certain animals and with certain substances it may, the pus being free from bacteria. It is still, however, questioned by some whether the pus thus produced really corresponds histologically and chemically with that due to bacterial action. Buchner showed that suppuration may be produced by the injection of dead bacteria, *e.g.* sterilised cultures of *bacillus pyocyaneus*, etc. The subject has now more a scientific than a practical interest, and the general statement may be made that practically all cases of true suppuration met with clinically are due to the action of living micro-organisms.

The term *septicæmia* is applied to conditions in which the organisms multiply within the blood and give rise to symptoms of general poisoning, without, however, producing abscesses in the organs. In all cases of septicæmia the organisms are more numerous in the capillaries of internal organs than in the peripheral circulation, and, in the case of the human subject, it may be impossible to detect any in the blood during life, though they may be seen in large numbers in the capillaries of the kidneys, liver, etc., *post mortem*. The essential fact in *pyæmia*, on the other hand, is the occurrence of multiple abscesses in internal organs and other parts of the body. In most of the cases of typical pyæmia, common in pre-antiseptic days, the starting-point of the disease was a septic wound with bacterial invasion of a vein leading to thrombosis and secondary embolism. Multiple foci of suppuration may be produced, however, in other ways, as will be described below (p. 186). If the term “pyæmia” be used to embrace all such conditions, their method of production should always be distinguished.



## BACTERIA AS CAUSES OF INFLAMMATION AND SUPPURATION.

A considerable number of species of bacteria have been found in acute inflammatory and suppurative conditions, and of these many have been proved to be causally related, whilst of some others the exact action has not yet been fully determined.

Ogston, who was one of the first to study this question (in 1881), found that the organisms most frequently present were micrococci, of which some were arranged irregularly in clusters (staphylococci), whilst others formed chains (streptococci). He found that the former were more common in circumscribed acute abscesses, the latter in spreading suppurative conditions. Rosenbach shortly afterwards (1884), by means of cultures, differentiated several varieties of micrococci, to which he gave the following special names: *staphylococcus pyogenes aureus*, *staphylococcus pyogenes albus*, *streptococcus pyogenes*, *micrococcus pyogenes tenuis*. Other organisms are met with in suppuration, such as *staphylococcus pyogenes citreus*, *staphylococcus cereus albus*, *staphylococcus cereus flavus*, *pneumococcus*, *pneumobacillus* (Friedländer), *bacillus pyogenes fœtidus* (Passet), *bacillus coli communis*, *bacillus lactis ærogenes*, *bacillus ærogenes encapsulatus*, *bacillus pyocyaneus*, *micrococcus tetragenus*, *pneumococcus*, *pneumobacillus*, *diplococcus intracellularis meningitidis*, and others.

In secondary inflammations and suppurations following acute diseases the corresponding organisms have been found in some cases, such as gonococcus, typhoid bacillus, influenza bacillus, etc. Suppuration is also produced by the actinomyces and the glanders bacillus, and sometimes chronic tubercular lesions have a suppurative character.

**Staphylococcus Pyogenes Aureus.**—*Microscopical Characters.*

—This organism is a spherical coccus about  $\cdot 9 \mu$  in diameter, which grows irregularly in clusters or masses (Fig. 52). It stains readily with all the basic aniline dyes, and retains the colour in Gram's method.

*Cultivation.*—It grows readily in all the ordinary media at the room temperature, though much more rapidly at the temperature of the body. In stab cultures in *peptone gelatin* a streak of growth is visible on the day after inoculation, and on the second or third day liquefaction commences at the top. As liquefaction proceeds, the growth falls to the bottom as a flocculent deposit, which soon assumes a bright yellow colour, while a yellowish film may form on the surface, the fluid portion still remaining turbid. Ultimately liquefaction extends out to

the wall of the tube (Fig. 53). In *gelatin plates* colonies may be seen with the low power of the microscope in twenty-four hours, as little balls somewhat granular on the surface and of brownish colour. On the second day they are visible to the

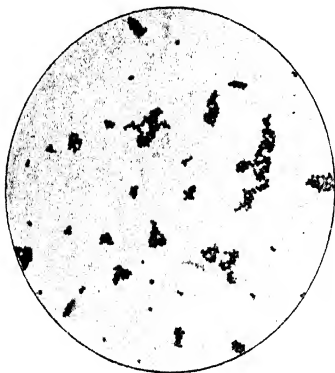


FIG. 52.—*Staphylococcus pyogenes aureus*, young culture on agar, showing clumps of cocci. Stained with weak carbol-fuchsin.  $\times 1000$ .

naked eye as whitish yellow points, which afterwards become more distinctly yellow. Liquefaction occurs around these, and little cups are formed, at the bottom of which the colonies form little yellowish masses. On *agar*, a stroke culture forms a line of abundant yellowish growth, with smooth, shining surface, well formed after twenty-four hours at  $37^{\circ}$  C. Later it becomes bright orange in colour, and resembles a streak of oil paint. Single colonies on the surface of agar are circular discs of similar appearance, which may reach 2 mm. or more in diameter. On *potatoes* it grows well at ordinary temperature, forming a somewhat abundant layer of orange colour. In *bouillon* it produces a uniform turbidity, which afterwards settles to the bottom as an abundant layer, which assumes a brownish yellow tint. In the various media it renders the reaction acid, and it coagulates



FIG. 53.—Two stab cultures of *staphylococcus pyogenes aureus* in gelatin, (a) 10 days old, (b) 3 weeks old, showing liquefaction of the medium and characters of growth. Natural size.

milk, in which it readily grows. The cultures have a somewhat sour odour.

It has considerable tenacity of life outside the body, cultures in gelatin often being alive after having been kept for several months. It also requires a rather higher temperature to kill it than most spore-free bacteria, viz. 80° C. for half an hour (Lübbert).

The *staphylococcus pyogenes albus* is similar in character, with the exception that its growth on all the media is white. The colour of the *staphylococcus aureus* may become less distinctly yellow after being kept for some time in culture, but it never assumes the white colour of the *staphylococcus albus*, and it has not been found possible to transform the one organism into the other. A micrococcus called by Welch *staphylococcus epidermidis albus* is practically always present in the skin epithelium; it is distinguished by its relatively non-pathogenic properties and by liquefying gelatin somewhat slowly. It is probably an attenuated variety of the *staphylococcus albus*.

The *staphylococcus pyogenes citreus*, which is less frequently met with, differs in the colour of the cultures being a lemon yellow, and is less virulent than the other two.

The *staphylococcus cereus albus* and *staphylococcus cereus flavus* are of much less importance. They produce a wax-like growth on gelatin without liquefaction; hence their name.

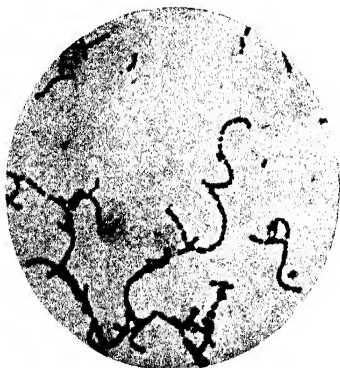


FIG. 54.—*Streptococcus pyogenes*, young culture on agar, showing chains of cocci. Stained with weak carbol-fuchsin.  $\times 1000$ .

**Streptococcus pyogenes.**—This organism is a coccus of slightly larger size than the *staphylococcus aureus* about 1  $\mu$  in diameter, and forms chains which may contain a large number of members, especially when it is growing

in fluids (Fig. 54). The chains vary somewhat in length in different specimens, and on this ground varieties have been distinguished, e.g. the *streptococcus brevis* and *streptococcus longus* (*vide infra*). As division may take place in many of the cocci

in a chain at the same time, the appearance of a chain of diplococci is often met with. In young cultures the cocci are fairly uniform in size, but after a time they present considerable variations, many swelling up to twice their normal diameter. These are to be regarded as involution forms. In its staining reactions the streptococcus resembles the staphylococci described, being readily coloured by Gram's method.

*Cultivation.*—In cultures outside the body the streptococcus pyogenes grows much more slowly than the staphylococci, and also

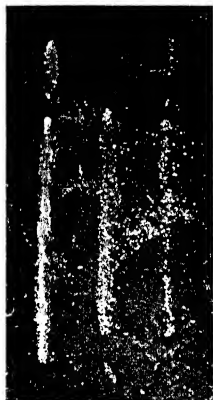


FIG. 55.—Culture of the streptococcus pyogenes on an agar plate, showing numerous colonies—three successive strokes. Twenty-four hours' growth. Natural size.



FIG. 56.—*Bacillus pyocyaneus*; young culture on agar. Stained with weak carbol-fuchsin.  $\times 1000$ .

dies out more readily, being in every respect a more delicate organism.

In *peptone gelatin* a stab culture shows, about the second day, a thin line, which in its subsequent growth is seen to be formed of a row of minute rounded colonies of whitish colour, which may be separate at the lower part of the puncture. They do not usually exceed the size of a small pin's head, this size being reached about the fifth or sixth day. The growth does not spread on the surface, and no liquefaction of the medium occurs. The colonies in gelatin plates have a corresponding appearance, being minute spherical points of whitish colour. A somewhat warm temperature is

necessary for growth ; even at 20° C. some varieties do not grow. On the *agar* media growth takes place along the stroke as a collection of small circular discs of semi-translucent appearance, which show a great tendency to remain separate (Fig. 55). The separate colonies remain small, rarely exceeding 1 mm. in diameter. Cultures on agar kept at the body temperature may often be found to be dead after ten days. On *potato*, as a rule, no visible growth takes place. In *milk* it produces a strongly acid reaction but no clotting of the medium. It ferments lactose, saccharose, and salicin (Andrewes and Horder) ; it produces no fermentation of inulin in Hiss's serum-water-medium, in this respect differing from the pneumococcus: It has a strong hæmolytic action, as can be demonstrated by growing it in blood-agar plates (p. 38). In *bouillon*, growth forms numerous minute granules which afterwards fall to the bottom, the deposit, which is usually not very abundant, having a sandy appearance. The appearance in broth, however, presents variations which have been used as an aid to distinguish different species of streptococci. It has been found that those which form the longest chains grow most distinctly in the form of spherical granules, those forming short chains giving rise to a finer deposit. To a variety which forms distinct spherules of minute size the term *streptococcus conglomeratus* has been given.

*Varieties of Streptococci.*—Formerly the *streptococcus pyogenes* and the *streptococcus erysipelatis* were regarded as two distinct species, and various points of difference between them were given. Further study, and especially the results obtained by modifying the virulence (p. 182), have shown that these distinctions cannot be maintained, and now practically all authorities are agreed that the two organisms are one and the same, erysipelas being produced when the streptococcus pyogenes of a certain standard of virulence gains entrance to the lymphatics of the skin. Petruschky, moreover, showed conclusively by inoculation that a streptococcus cultivated from pus could cause erysipelas in the human subject.

Streptococci have also been classified according to the length of the chains. Thus there have been distinguished (*a*) *streptococcus longus*, which occurs in long chains and is pathogenic to rabbits and mice ; (*b*) *streptococcus brevis*, which is common in the mouth in normal conditions, and is usually non-pathogenic ; and (*c*) *streptococcus conglomeratus*, so called from its forming in bouillon minute granules composed of very long chains. It may be stated that pathogenic streptococci obtained from the human subject usually form fairly long chains on agar, whilst the short

streptococci obtained from the mouth and intestine are usually devoid of virulence. But to these statements exceptions occur, as short streptococci may be associated with grave lesions; it has also been found that the length of the chains is not a constant feature. As in the case of other organisms attempts have also been made to differentiate streptococci by means of their fermentative properties. Mervyn Gordon introduced for this purpose nine tests, namely:—(1) The clotting of milk, (2) the reduction of neutral red, (3-9) the fermentation with acid production of saccharose, lactose, raffinose, inulin, salicin, coniferin, and mannite. Andrewes and Horder by means of these have differentiated six varieties, of which five occur in the human subject. These are:—(a) A short-chained form called *streptococcus mitis*, which occurs chiefly in the saliva and fæces as a saprophyte. (b) The *streptococcus pyogenes*, which is the most important pathogenic variety, and has the characters described above. (c) The *streptococcus salivarius*, which corresponds to the streptococcus brevis of the mouth, and which, as regards fermentative action, seems to bear the same relation to the next variety as the streptococcus mitis does to the streptococcus pyogenes. It has more active fermentative properties and clots milk. (d) The *streptococcus anginosus*, which corresponds with the so-called streptococcus scarlatinae and the streptococcus conglomeratus. It usually clots milk and does not grow on gelatin at 20° C. (e) The *streptococcus faecalis*, a short-chained form, which abounds in the intestine and which has great fermentative activity. It forms sulphuretted hydrogen, and is devoid of hæmolytic action. (f) The sixth variety is the *streptococcus equinus*, which is common in the air and dust of towns, and appears to be derived from horse dung.<sup>1</sup>

Schottmüller has employed the appearance of the colonies of streptococci on blood agar as a means of separating varieties, the medium used consisting of two parts human blood and five parts melted agar. He distinguishes the *streptococcus longus* or *erysipclatis*, which forms grey colonies and has a hæmolytic action; a *streptococcus mitior* or *viridans*, a short-chained organism, which produces small green colonies and very little hæmolysis, and a *streptococcus mucosus encapsulatus*, which, as its name indicates, shows well-marked capsules and produces colonies which have a slimy consistence. It should be noted that on blood agar the pneumococcus forms green colonies and produces no hæmolysis.

<sup>1</sup> For further details reference must be made to the original papers, *Lancet*, September 1906, ii. 708, etc.

It will be thus seen from this account that the streptococcus pyogenes as described above is the organism most frequently associated with the pathogenic processes, and that short-chained forms are common saprophytes in the human body, although they may be associated with conditions of disease; these may be subdivided according to their fermentative activity as detailed. And lastly, there is the streptococcus conglomeratus (anginosus), which is specially abundant in the throat in scarlet fever, though it also occurs in other acute catarrhal states. No definite statement can yet be made as to the etiological relation of streptococci to scarlet fever; we can only say that streptococci are almost invariably present in the fauces, and that to them many of the complications of the disease are due.

**Bacillus coli communis.**—The microscopic and cultural characters are described in the chapter on typhoid fever. The *bacillus lactis arogenes* and the *bacillus pyogenes fetidus* closely resemble it; they are either varieties or closely related species. The former is distinguished by producing more abundant gas formation, and by its growth on gelatin, etc., being thicker and whiter than that of the bacillus coli.

**Bacillus ærogenes encapsulatus** sometimes invades the tissues before death, and is characterised by the formation of bubbles of gas in the infected parts. Its characters are described in Chapter XVI.

**Bacillus pyocyaneus.**—This organism occurs in the form of minute rods 1·5 to 3  $\mu$  in length and less than ·5  $\mu$  in thickness (Fig. 56). Occasionally two or three are found attached end to end. They are actively motile, and do not form spores. They stain readily with the ordinary basic stains, but are decolorised by Gram's method.

**Cultivation.**—It grows readily on all the ordinary media at the room temperature, the cultures being distinguished by the formation of a greenish pigment. In puncture cultures in peptone-gelatin a greyish line appears in twenty-four hours, and at its upper part a small cup of liquefaction forms within forty-eight hours. At this time a slightly greenish tint is seen in the superficial part of the gelatin. The liquefaction extends pretty rapidly, the fluid portion being turbid and showing masses of growth at its lower part. The green colour becomes more and more marked and diffuses through the gelatin. Ultimately liquefaction reaches the wall of the tube. In plate cultures the colonies appear as minute whitish points, those on the surface being the larger. Under a low power of the microscope they have a brownish-yellow colour and show a nodulated surface, the superficial colonies being thinner and larger. Liquefaction soon occurs, the colonies on the surface forming shallow cups with small irregular masses of growth at the bottom, the deep colonies small spheres of liquefaction. Around the colonies a greenish tint appears. On agar the growth forms an abundant slimy greyish layer which afterwards becomes greenish, and a bright green colour diffuses through the whole substance of the medium. On potatoes the growth is an abundant reddish-brown layer resembling that of the glanders bacillus, and the potato sometimes shows a greenish discoloration.

From the cultures there can be extracted by chloroform a coloured body pyocyanin, which belongs to the aromatic series, and crystallises

in the form of long, delicate bluish-green needles. On the addition of a weak acid its colour changes to a red.

This organism has distinct pathogenic action in certain animals. Subcutaneous injection of small doses in rabbits may produce a local suppuration, but if the dose be large, spreading hæmorrhagic œdema results, which may be attended by septicæmia. Intravenous injection may produce, according to the dose, rapid septicæmia with nephritis, or sometimes a more chronic condition of wasting attended by albuminuria.

***Micrococcus tetragenus.***

—This organism, first described by Gaffky, is characterised by the fact that it divides in two planes at right angles to one another (Fig. 57), and is thus generally found in the tissues in groups of four or tetrads, which are often seen to be surrounded by a capsule. The cocci measure  $1\ \mu$  in diameter. They stain readily with all the ordinary stains, and also retain the stain in Gram's method.

It grows readily on all the media at the room temperature. In a puncture culture on peptone-gelatin a pretty thick whitish line forms along the track of the needle, whilst on the surface there is a thick rounded disc of whitish colour. The gelatin is not liquefied. On the surface of agar and of potato the growth is an abundant moist layer of the same colour. The growth on all the media has a peculiar viscid or tenacious character, owing to the gelatinous character of the sheaths of the cocci.

White mice are exceedingly susceptible to this organism. Subcutaneous injection is followed by a general septicæmia, the organism being found in large numbers in the blood throughout the body. Guinea-pigs are less susceptible; sometimes only a local abscess with a good deal of necrotic change results; sometimes there is also septicæmia.

**Experimental Inoculation.**—We shall consider chiefly the *staphylococcus pyogenes aureus* and the *streptococcus pyogenes*, as these have been most fully studied.

It may be stated at the outset that the occurrence of suppuration depends upon the number of organisms introduced into the tissues, the number necessary varying not only in different animals, but also in different parts of the same animal,—a smaller number producing suppuration in the anterior chamber of the eye, for example, than in the peritoneum. The virulence of the

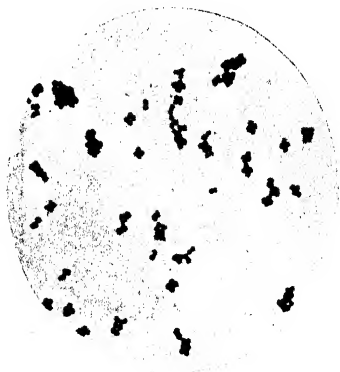


FIG. 57.—*Micrococcus tetragenus*; young culture on agar, showing tetrads. Stained with weak carbol-fuchsin.  $\times 1000$ .



organism also may vary, and corresponding results may be produced. Especially is this so in the case of the *streptococcus pyogenes*.

The *staphylococcus aureus*, when injected *subcutaneously* in suitable numbers, produces an acute local inflammation, which is followed by suppuration, in the manner described above. The spread of the suppuration goes *pari passu* with the growth of the cocci. If a large dose is injected the cocci may enter the blood stream in sufficient numbers to cause secondary suppurative foci in internal organs (*cf.* intravenous injection).

*Intravenous injection* in rabbits, for example, produces interesting results which vary according to the quantity used. If a considerable quantity be injected, the animal may die in twenty-four hours of a general septicæmia, numerous cocci being present in the capillaries of the various organs, often forming plugs. If a smaller quantity be used, the cocci gradually disappear from the circulating blood; some become destroyed, while others settle in the capillary walls in various parts and produce minute abscesses. These are most common in the kidneys, where they occur both in the cortex and medulla as minute yellowish areas surrounded by a zone of intense congestion and hæmorrhage. Similar small abscesses may be produced in the heart wall, in the liver, under the periosteum, and in the interior of bones, and occasionally in the striped muscles. Very rarely indeed, in experimental injection, do the cocci settle on the healthy valves of the heart. If, however, when the organisms are injected into the blood, there be any traumatism of a valve, or of any other part of the body, they show a special tendency to settle at these weakened points.

Experiments on the *human subject* have also proved the pyogenic properties of those organisms. Garré inoculated scratches near the root of his finger-nail with a pure culture, a small cutaneous pustule resulting; and by rubbing a culture over the skin of the forearm he caused a carbuncular condition which healed only after some weeks. Confirmatory experiments of this nature have been made by Bockhart, Bumm, and others.

When tested experimentally the *staphylococcus pyogenes albus* has practically the same pathogenic effects as the *staphylococcus aureus*.

The *streptococcus pyogenes* is an organism the virulence of which varies much according to the diseased condition from which it has been obtained, and also one which loses its virulence rapidly in cultures. Even highly virulent cultures, if grown under ordinary conditions, in the course of time lose practically all

pathogenic power. By passage from animal to animal, however, the virulence may be much increased, and *pari passu* the effects of inoculation are correspondingly varied. Marmorek, for example, found that the virulence of a streptococcus can be enormously increased by growing it alternately (*a*) in a mixture of human blood serum and bouillon (*vide* page 41), and (*b*) in the body of a rabbit; ultimately, after several passages it possesses a super-virulent character, so that even an extremely minute dose introduced into the tissues of a rabbit produces rapid septicæmia, with death in a few hours. It has been proved by Marmorek's experiments, and those of others, that the same species of streptococcus may produce at one time merely a passing local redness, at another a local suppuration, at another a spreading erysipelatous condition, or again a general septicæmic infection, according as its virulence is artificially increased. Such experiments are of extreme importance as explaining to some extent the great diversity of lesions in the human subject with which streptococci are associated.

**Bacillus Coli Communis.**—The virulence of this organism also varies much and can be increased by passage from animal to animal. Injection into the serous cavities of rabbits produces a fibrinous inflammation which becomes purulent if the animal lives sufficiently long. If, however, the virulence of the organism be of a high order, death takes place before suppuration is established, and there is a septicæmic condition, the organisms occurring in large numbers in the blood. Intravenous injection of a few drops of a virulent bouillon culture usually produces a rapid septicæmia with scattered hæmorrhages in various organs.

**Other Effects.**—It has been found by independent observers that in cases where rabbits recover after intravenous injection of bacillus coli communis, a certain proportion suffer from paralysis and sometimes from atrophy of muscles, especially of the posterior limbs, these symptoms being due to lesions of the cells in the anterior cornua of the spinal cord. Somewhat similar results have been obtained by others after inoculations with staphylococci and streptococci, a certain proportion only of the animals showing paralytic symptoms and corresponding changes in the spinal cord. The lesions are believed to be due chiefly to the action of the products of the organisms on the highly organised nervous elements. Much further research requires to be done before the importance of these results can be properly estimated, but it is not improbable that they will throw light on the causation of nervous lesions which occur in the human subject, and the etiology of which at present is quite obscure. Some observers, chiefly of the French school, consider that paralysis associated with cystitis, in which the bacillus coli communis is often present, may have such a causation, and that paralytic conditions following acute infective fevers may be produced by the products of pyogenic cocci, which frequently occur in these conditions.

•

**Lesions in the Human Subject.**—The following statement may be made with regard to the occurrence of the chief organisms mentioned, in the various suppurative and inflammatory conditions in the human subject. The account is, however, by no means exhaustive, as clinical bacteriology has shown that practically every part of the body may be the site of a lesion produced by the pyogenic bacteria. It may also be noted that acute catarrhal conditions of cavities or tubes are in many cases also to be ascribed to their presence.

The *staphylococci* are the most common causal agents in localised abscesses, in pustules on the skin, in carbuncles, boils, etc., in acute suppurative periostitis, in catarrhs of mucous surfaces, in ulcerative endocarditis, and in various pyæmic conditions. They may also be present in septicaemia.

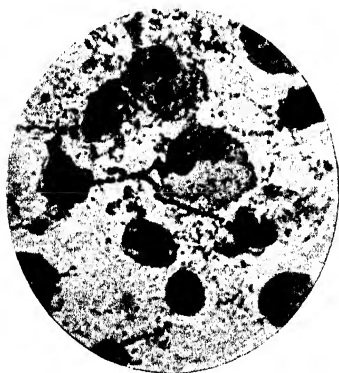


FIG. 58.—Streptococci in acute suppuration. Corrosive film; stained by Gram's method and safranin.  $\times 1000$ .

*Streptococci* are especially found in spreading inflammation with or without suppuration; in diffuse phlegmonous and erysipelatous conditions, suppurations in serous membranes and in joints (Fig. 58). They also occur in acute suppurative periostitis and ulcerative endocarditis. Secondary abscesses in lymphatic

glands and lymphangitis are also, we believe, more frequently caused by streptococci than staphylococci. They also produce fibrinous exudation on the mucous surfaces, leading to the formation of false membrane in many of the cases of non-diphtheritic inflammation of the throat, which are met with in scarlatina<sup>1</sup> and other conditions, and they are also the organisms most frequently present in acute catarrhal inflammations in this situation. In puerperal peritonitis they are frequently found in a condition of purity, and they also appear to be the most frequent cause of puerperal septicaemia, in which condition they may be found after death in the capillaries of various organs.

<sup>1</sup> True diphtheria may also occasionally be associated with this disease, usually as a sequel.

In pyæmia they are frequently present, though in most cases associated with other pyogenic organisms. Some cases of enteritis in infants—streptococcic enteritis—are also apparently due to a streptococcus, which, however, presents in cultures certain points of difference from the streptococcus pyogenes.

The *bacillus coli communis* is found in a great many inflammatory and suppurative conditions in connection with the alimentary tract—for example, in suppuration in the peritoneum, or in the extraperitoneal tissue with or without perforation of the bowel, in the peritonitis following strangulation of the bowel, in appendicitis and the lesions following it, in suppuration around the bile-ducts, etc. It may also occur in lesions in other parts of the body,—endocarditis, pleurisy, etc., which in some cases are associated with lesions of the intestine, though in others such cannot be found. It is also frequently present in inflammation of the urinary passages, cystitis, pyelitis, abscesses in the kidneys, etc., these lesions being in fact most frequently caused by this or closely allied organisms.

In certain cases of enteritis it is probably the causal agent, though this is difficult of proof, as it is much increased in numbers in practically all abnormal conditions of the intestine. We may remark that it has been repeatedly proved that the bacillus coli cultivated from various lesions is more virulent than that in the intestine, its virulence having been heightened by growth in the tissues.

The *micrococcus tetragenus* is often found in suppurations in the region of the mouth or in the neck, and also occurs in various lesions of the respiratory tract, in phthisical cavities, abscesses in the lungs, etc. Sometimes it is present alone, and probably has a pyogenic action in the human subject under certain conditions. In other cases it is associated with other organisms. Recently one or two cases of pyæmia have been described in which this organism was found in a state of purity in the pus in various situations. In this latter condition the pus has been described as possessing an oily, viscous character, and as being often blood-stained.

The *bacillus pyocyaneus* is rarely found alone in pus, though it is not infrequent along with other organisms. We have met with it twice in cases of multiple abscesses, in association with the staphylococcus pyogenes aureus. Lately some diseases in children have been described in which the bacillus pyocyaneus has been found throughout the body; in these cases the chief symptoms have been fever, gastro-intestinal irritation, pustular or petechial eruptions on the skin, and general marasmus.

Suppurative and inflammatory conditions, associated with the organisms of special diseases, will be described in the respective chapters.

**Mode of Entrance and Spread.**—Many of the organisms of suppuration have a wide distribution in nature, and many also are present on the skin and mucous membranes of healthy individuals. Staphylococci are commonly present on the skin,

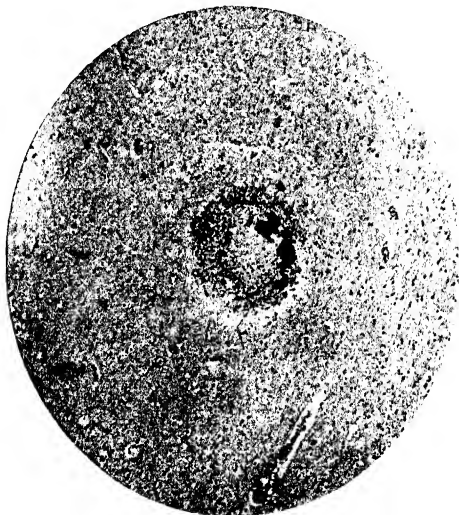


FIG. 59.—Minute focus of commencing suppuration in brain—case of acute ulcerative endocarditis. In the centre a small hæmorrhage; to right side dark masses of staphylococci; zone of leucocytes at periphery.

Alum carmine and Gram's method.  $\times 50$ .

and also occur in the throat and other parts, and streptococci can often be cultivated from the secretions of the mouth in normal conditions. The pneumococcus of Fraenkel and the pneumobacillus of Friedländer have also been found in the mouth and in the nasal cavity, whilst the bacillus coli communis is a normal inhabitant of the intestinal tract. The entrance of these organisms into the deeper tissues when a surface lesion occurs can be readily understood. Their action will, of course, be favoured by any condition of depressed vitality. Though in normal conditions the blood is bacterium-free, we must suppose

that from time to time a certain number of such organisms gain entrance to it from trifling lesions of the skin or mucous surfaces, the possibilities of entrance from the latter being especially numerous. In most cases they are killed by the action of the healthy serum or cells of the body, and no harm results. If, however, there be a local weakness, they may settle in that part

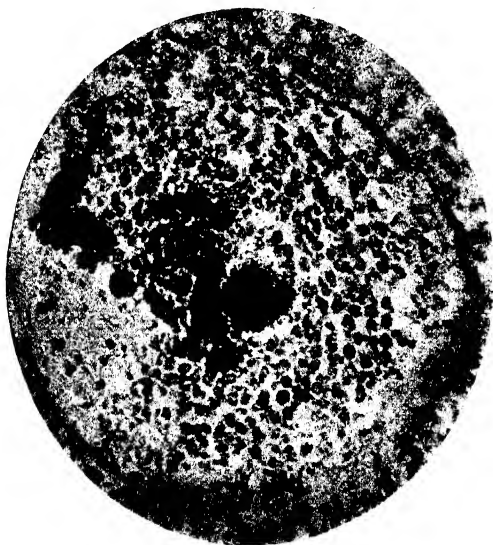


FIG. 60.—Secondary infection of a glomerulus of kidney by the staphylococcus aureus, in a case of ulcerative endocarditis. The cocci (stained darkly) are seen plugging the capillaries and also lying free. The glomerulus is much swollen, infiltrated by leucocytes, and partly necrosed. Paraffin section; stained by Gram's method and Bismarek-brown.  $\times 300$ .

and produce suppuration, and from this other parts of the body may be infected. Such a supposition as this is necessary to explain many inflammatory and suppurative conditions met with clinically. In some cases of multiple suppurations due to staphylococcus infection, which we have had the opportunity to examine, only an apparently unimportant surface lesion was present; whilst in others no lesion could be found to explain the origin of the infection. The term *cryptogenetic* has been applied by some writers to such cases in which the original

point of infection cannot be found, but its use is scarcely necessary.

The paths of secondary infection may be conveniently summarised thus: First, by lymphatics. In this way the lymphatic glands may be infected, and also serous sacs in relation to the organs where the primary lesion exists. Second, by natural channels, such as the ureters and the bile-ducts, the spread being generally associated with an inflammatory condition of the lining epithelium. In this way the kidneys and liver respectively may be infected. Third, by the blood vessels: (a) by a few organisms gaining entrance to the blood from a local lesion, and settling in a favourable nidus or a damaged tissue, the original path of infection often being obscure; (b) by a septic phlebitis with suppurative softening of the thrombus and resulting embolism; and we may add (c) by a direct extension along a vein, producing a spreading thrombosis and suppuration within the vein. In this way suppuration may spread along the portal vein to the liver from a lesion in the alimentary canal, the condition being known as *pylephlebitis suppurativa*.

Although many of the lesions produced by the bacteria under consideration have already been mentioned, certain conditions may be selected for further consideration on account of their clinical importance or bacteriological interest.

**Endocarditis.**—There is now strong presumptive evidence that all cases of endocarditis are due to bacterial infection. In the simple or vegetative form, so often the result of acute rheumatism, the *micrococcus rheumaticus* (p. 193) has been cultivated from the valves in a certain number of cases, and is probably the causal agent in most instances.

Endocarditis of the ulcerative type may be produced by various organisms, chiefly pyogenic. Of these the staphylococci and streptococci are most frequently found. In some cases of ulcerative endocarditis following pneumonia, the pneumococcus (Fraenkel's) is present; in others pyogenic cocci, especially streptococci. Other organisms have been cultivated from different cases of the disease, and some of these have received special names; for example, the *diplococcus endocarditis encapsulatus*, *bacillus endocarditidis griseus* (Weichselbaum), and others. In some cases the *bacillus coli communis* has been found, and occasionally in endocarditis following typhoid the typhoid bacillus has been described as the organism present, but further observations on this point are desirable. The gonococcus also has been shown to affect the heart valves (p. 225), though this is a very rare occurrence. Tubercle nodules on the heart valves

have been found in a few cases of acute tuberculosis, though no vegetative or ulcerative condition is produced.

In some cases, though we believe not often, the organisms may attack healthy valves, producing a *primary* ulcerative endocarditis, but more frequently the valves have been the seat of previous endocarditis, *secondary* ulcerative endocarditis being

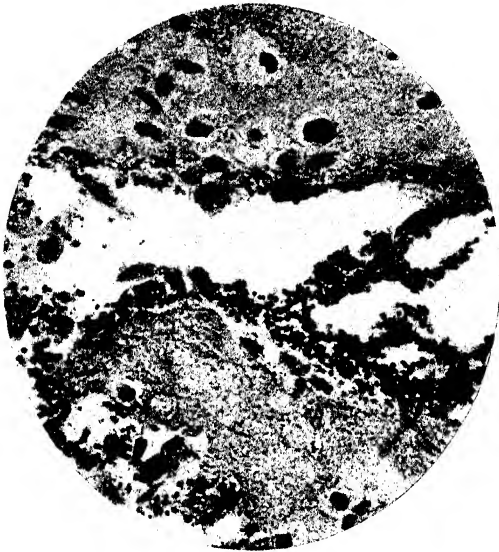


FIG. 61.—Section of a vegetation in ulcerative endocarditis, showing numerous staphylococci lying in the spaces. The lower portion is a fragment in process of separation.

Stained by Gram's method and Bismarck-brown.  $\times 600$ .

thus produced. In some cases, especially when the valves have been previously diseased, the source of the infection is quite obscure. It is evident that as the vegetations are composed for the most part of unorganised material, they do not offer the same resistance to the growth of bacteria, when a few reach them, as a healthy cellular tissue does. On microscopic examination of the diseased valves the organisms are usually to be found in enormous numbers, sometimes forming an almost continuous layer on the surface, or occurring in large masses or clusters in spaces in the vegetation (Fig. 61). By their action a certain amount



of softening or breaking down of the vegetations occurs, and the emboli thus produced act as the carriers of infection to other organs, and give rise to secondary suppurations.

*Experimental.*—Occasionally ulcerative endocarditis is produced by the simple intravenous injection of staphylococci and streptococci into the circulation, but this is a very rare occurrence. It often follows, however, when the valves have been previously injured. Orth and Wyssokowitsch at a comparatively early date produced the condition by damaging the aortic cusps by a glass rod introduced through the carotid, and afterwards injecting staphylococci into the circulation. Similar experiments have since been repeated with streptococci, pneumococci, and other organisms, with like result. Ribbert found that if a potato culture of the staphylococcus aureus were rubbed down in salt solution so as to form an emulsion, and then injected into the circulation, some minute fragments became arrested at the attachment of the chordæ tendineæ and produced an ulcerative endocarditis.

**Acute Suppurative Periostitis and Osteomyelitis.**—Special mention is made of this condition on account of its comparative frequency and gravity. The great majority of cases are caused by the pyogenic cocci, of which one or two varieties may be present, the staphylococcus aureus, however, occurring most frequently. Pneumococci have been found alone in some cases, and in a few cases following typhoid fever, apparently well authenticated, the typhoid bacillus has been found alone. In others again the bacillus coli communis is present.

The affection of the periosteum or interior of the bones by these organisms, which is especially common in young subjects, may take place in the course of other affections produced by the same organisms or in the course of infective fevers, but in a great many cases the path of entrance cannot be determined. In the course of this disease serious secondary infections are always very liable to follow, such as small abscesses in the kidneys, heart-wall, lungs, liver, etc., suppurations in serous cavities, and ulcerative endocarditis; in fact, some cases present the most typical examples of extreme general staphylococcus infection. The entrance of the organisms into the blood stream from the lesion of the bone is especially favoured by the arrangement of the veins in the bone and marrow.

*Experimental.*—Multiple abscesses in the bones and under the periosteum may occur in simple intravenous injection of the pyogenic cocci into the blood, and are especially liable to be formed when young animals are used. These abscesses are of small size, and do not spread in the same way as in the natural disease in the human subject.

In experiments on healthy animals, however, the conditions are not analogous to those of the natural disease. We must presume that in the latter there is some local weakness or susceptibility which enables the

few organisms which have reached the part by the blood to settle and multiply. Moreover, if a bone be experimentally injured, *e.g.* by actual fracture or by stripping off the periosteum, before the organisms are injected, then a much more extensive suppuration occurs at the injured part.

**Erysipelas.**—A spreading inflammatory condition of the skin may be produced by a variety of organisms, but the disease in the human subject in its characteristic form is almost invariably due to a streptococcus, as was shown by Fehleisen in 1884. He obtained pure cultures of the organism, and gave it the name of *streptococcus erysipelatis*; and, further, by inoculations on the human subject as a therapeutic measure in malignant disease, he was able to reproduce erysipelas. As stated above, however, one after another of the supposed points of difference between the streptococcus of erysipelas and that of suppuration has broken down, and it is now generally held that erysipelas is produced by the streptococcus pyogenes of a certain degree of virulence. It must be noted, however, that erysipelas passes from patient to patient as erysipelas, and purulent conditions due to streptococci do not appear liable to be followed by erysipelas. On the other hand, the connection between erysipelas and puerperal septicæmia is well established clinically.

In a case of erysipelas the streptococci are found in large numbers in the lymphatics of the cutis and underlying tissues, just beyond the swollen margin of the inflammatory area. As the inflammation advances they gradually die out, and after a time their extension at the periphery comes to an end. The streptococci may extend to serous and synovial cavities and set up inflammatory or suppurative change,—peritonitis, meningitis, and synovitis may thus be produced.

**Conjunctivitis.**—A considerable number of organisms are concerned in the production of conjunctivitis and its associated lesions. Of these a number appear to be specially associated with this region. Thus a small organism, generally known as the Koch-Weeks bacillus, is the most common cause of acute contagious conjunctivitis, especially prevalent in Egypt, but also common in this country. This organism is very minute, being little more than  $1\ \mu$  in length, and morphologically resembles the influenza bacillus; its conditions of growth are even more restricted, as it rarely grows on blood agar, the best medium being serum agar. On this medium it produces minute transparent colonies like drops of dew. The obtaining of pure cultures is a matter of considerable difficulty, and it is nearly always accompanied by the xerosis bacillus. It can readily be

found in the muco-purulent secretion by staining films with weak (1:10) carbolfuchsin, and is often to be seen in the interior of leucocytes (Fig. 62).



FIG. 62.—Film preparation from a case of acute conjunctivitis, showing Koch-Weeks bacilli, chiefly contained within a leucocyte. (From a preparation by Dr. Inglis Pollock.)  
× 1000.

Another organism exceedingly like the previous, apparently differing from it only in the rather wider conditions of growth, is Müller's bacillus. It has been cultivated by him in a considerable proportion of cases of trachoma, but its relation to this condition is still matter of dispute. Another bacillus which is now well recognised is the diplo-bacillus of conjunctivitis

first described by Morax. It is especially common in the more subacute cases of conjunctivitis. Eyre found it in 2·5 per cent of all cases of conjunctivitis. Its cultural characters are given below. The xerosis bacillus, which is a small diphtheroid organism (Fig. 123), has been found in xerosis of the conjunctiva, in follicular conjunctivitis, and in other conditions; it appears to occur sometimes also in the normal conjunctiva. It is doubtful whether it has any pathogenic action of importance. Acute conjunctivitis is also produced by the pneumococcus, epidemics of the disease being sometimes due to this organism, and also by



FIG. 63.—Film preparation of conjunctival secretion showing the Morax diplo-bacillus of conjunctivitis. × 1000.

streptococci and staphylococci. True diphtheria of the conjunctiva caused by the Klebs-Löffler bacillus also occurs, whilst in gonorrhoeal conjunctivitis, often of an acute purulent type, the gonococcus is present (p. 225).

**Diplo-bacillus of Conjunctivitis.**—This organism, discovered by Morax, is a small plump bacillus, measuring  $1 \times 2 \mu$ , and usually occurring in pairs, or in short chains of pairs (Fig. 63). It is non-motile, does not form spores, and is decolorised by Gram's method. It does not grow on the ordinary gelatin and agar media, the addition of blood or serum being necessary. On serum it forms small rounded colonies which produce small pits of liquefaction; hence it has been called the *bacillus lacunatus*. In cultures it is distinctly pleomorphic, and involution forms also occur. It is non-pathogenic to the lower animals.

**Acute Rheumatism.**—There are many facts which point to the infective nature of this disease, and investigations from this point of view have yielded important results. Of the organism isolated, the one which appears to have strongest claims is a small coccus observed by Triboulet, and by Westphal and Wassermann, the characters and action of which were first investigated in this country by Poynton and Paine. It is now usually spoken of as the *micrococcus rheumaticus*. The organism is sometimes spoken of as a diplococcus, but it is best described as a streptococcus growing in short chains; in the tissues, however, it usually occurs in pairs. It is rather smaller than the streptococcus pyogenes, and although it can be stained by Gram's method, it loses the colour more readily than the streptococcus. In the various media it produces a large amount of acid, and usually clots milk after incubation for two days; on blood agar it alters the hæmoglobin to a brownish colour. Its growth on media generally is more luxuriant than that of the streptococcus, and it grows well on gelatin at  $20^{\circ}$  C. Intravenous injection of pure cultures in rabbits often produces polyarthritis and synovitis, valvulitis and pericarditis, without any suppurative change—lesions which it is stated are not produced by the ordinary streptococci (Beattie). In one or two instances choreiform movements have been observed after injection. The organism is most easily obtained from the substance of inflamed synovial membrane where it is invading the tissues; a part where there is special congestion should be selected as being most likely to give positive results. It is only occasionally to be obtained from the fluid in joints. It has also been cultivated from the blood in rheumatic fever, from the vegetations on the heart valves, and from other acute lesions; in many cases, however, cultures from the blood give negative results. Poynton

and Paine cultivated it from the cerebro-spinal fluid in three cases where chorea was present, and also detected it in the membranes of the brain. They consider that this disease is probably of the nature of a slight meningo-mylitis produced by this organism. The facts already accumulated speak strongly in favour of this organism being causally related to rheumatic fever, though this cannot be considered completely proved. Andrewes finds that the organism has the same cultural characters and fermentative effects as the *streptococcus faecalis*, a common inhabitant of the intestine. Even, however, if the two organisms were the same, it might well be possible that rheumatic fever is due to an infection of the tissues by this variety of streptococcus. The clinical data, in fact, rather point to rheumatic fever being due to an infection by some organism frequently present in the body, brought about by some state of predisposition or acquired susceptibility.

#### **Vaccination Treatment of Infections by the Pyogenic Cocci.**

—From his study of the part played by phagocytosis in the successful combat of the pyogenic bacteria by the body, Wright was led to advocate the treatment of such infections by the origination during their course of an active immunisation by dead cultures of the infecting agent. The treatment is applicable when the infection is practically local as in acne pustules, in boils, etc. (For the theoretical questions raised see Immunity.) It is best to attempt to isolate the causal organism from the lesion and to test the opsonic index of the patient against it. To prepare the vaccine an agar slope culture is taken and the growth washed off with normal saline. The organism is then killed by steaming for an appropriate time, and the efficacy of the sterilisation tested by inoculating fresh agar tubes. The strength of the emulsion is estimated by the method of counting dead bacteria described on p. 67. The number of bacteria used for an injection is from 250,000,000 to 500,000,000, and in the details of the measurement of this quantity and in its injection every aseptic precaution must, of course, be adopted. If repeated injections are necessary Wright recommends that the opsonic index should be observed every few days and the injections only practised during a positive phase. If it is not practicable to use the infecting strain for the preparation of the vaccine, then laboratory cultures must be used, and in such cases it is well to use a mixture of strains; in skin infections a mixture of *staphylococcus aureus* and *albus* may be employed. Such means have been extensively used in the treatment of acne, boils, sycosis, infections of the genito-urinary tract by the

*b. coli*, infections of joints by the gonococcus, and in many cases considerable success has followed the treatment.

**Methods of Examination in Inflammatory and Suppurative Conditions.**—These are usually of a comparatively simple nature, and include (1) microscopic examination, (2) the making of cultures.

(1) The pus or other fluids should be examined microscopically, first of all by means of film preparations in order to determine the characters of the organisms present. The films should be stained (*a*) by one of the ordinary solutions, such as carbol-thionin-blue (p. 98), or a saturated watery solution of methylene-blue; and (*b*) by Gram's method. The use of the latter is of course of high importance as an aid in the recognition.

(2) As most of the pyogenic organisms grow readily on the gelatin media at ordinary temperatures, pure cultures can be readily obtained by the ordinary plate methods. But in many cases the separation can be effected much more rapidly by the method of successive streaks on agar tubes, which are then incubated at 37° C. When the presence of pneumococci is suspected this method ought always to be used, and it is also to be preferred in the case of streptococci. Inoculation experiments may be carried out as occasion arises.

In cases of suspected blood infection the examination of the blood is to be carried out by the methods already described (p. 68).

## CHAPTER VII.

### INFLAMMATORY AND SUPPURATIVE CONDITIONS, *CONTINUED*: THE ACUTE PNEUMONIAS, EPI- DEMIC CEREBRO-SPINAL MENINGITIS.

**Introductory.**—The term Pneumonia is applied to several conditions which present differences in pathological anatomy and in origin. All of these, however, must be looked on as varieties of inflammation in which the process is modified in different ways depending on the special structure of the lung or of the parts which compose it. There is, first of all, and, in adults, the commonest type, the acute croupous or lobar pneumonia, in which an inflammatory process attended by abundant fibrinous exudation affects, by continuity, the entire tissue of a lobe or of a large portion of the lung. It departs from the course of an ordinary inflammation in that the reaction of the connective tissue of the lung is relatively slight, and there is usually no tendency for organisation of the inflammatory exudation to take place. Secondly, there is the acute catarrhal or lobular pneumonia, where a catarrhal inflammatory process spreads from the capillary bronchi to the air vesicles, and in these a change, consisting largely of proliferation of the endothelium of the alveoli, takes place which leads to consolidation of patches of the lung tissue. Up till 1889 acute catarrhal pneumonia was comparatively rare except in children. In adults it was chiefly found as a secondary complication to some condition such as diphtheria, typhoid fever, etc. Since, however, influenza in an epidemic form has become frequent, catarrhal pneumonia has been of much more frequent occurrence in adults, has assumed a very fatal tendency, and has presented the formerly quite unusual feature of being sometimes the precursor of gangrene of the lung. Besides these two definite types other forms also occur. Thus instead of a fibrinous material the exudation may

be of a serous, hæmorrhagic, or purulent character. Cases of mixed fibrinous and catarrhal pneumonia also occur, and in the catarrhal there may be great leucocytic emigration. Hæmorrhages also may occur here.

Besides the two chief types of pneumonia there is another group of cases which are somewhat loosely denominated septic pneumonias, and which may arise in two ways: (1) by the entrance into the trachea and bronchi of discharges, blood, etc., which form a nidus for the growth of septic organisms; these often set up a purulent capillary bronchitis and lead to infection of the air cells and also of the interstitial tissue of the lung; (2) from secondary pyogenic infection by means of the blood stream from suppurative foci in other parts of the body. (See chapter on Suppuration, etc.) In these septic pneumonias various changes, resembling those found in the other types, are often seen round the septic foci.

In pneumonias, therefore, there may be present a great variety of types of inflammatory reaction. We shall see that with all of them bacteria have been found associated. Special importance is attached to acute croupous pneumonia on account of its course and characters, but reference will also be made to the other forms.

**Historical.**—Acute lobar pneumonia for long was supposed to be an effect of exposure to cold; but many observers were dissatisfied with this view of its etiology. Not only did cases occur where no such exposure could be traced, but it had been observed that the disease sometimes occurred epidemically, and was occasionally contracted by hospital patients lying in beds adjacent to those occupied by pneumonia cases. Further, the sudden onset and definite course of the disease conformed to the type of an acute infective fever; it was thus suspected by some to be due to a specific infection. This view of its etiology was promulgated in 1882-83 by Friedländer, whose results were briefly as follows. In pneumonic lungs there were cocci, adherent usually in pairs, and possessed of a definitely contoured capsule. These cocci could be isolated and grown on gelatin, and on inoculation in mice they produced a kind of septicæmia with inflammation of the serous membranes. The blood and the exudation in serous cavities contained numerous capsulated diplococci. There is little doubt that many of the organisms seen by Friedländer were really Fraenkel's pneumococcus, to be presently described.

By many observers it had been found that the sputum of healthy men, when injected into animals, sometimes caused death, with the same symptoms as in the case of the injection of Friedländer's coccus; and in the blood and serous exudations of such animals capsulated diplococci were found. A. Fraenkel found that the sputum of pneumonic patients was much more fatal and more constant in its effects than that of healthy individuals. The cocci which were found in animals dead of this "sputum septicæmia," as it was called, differed from Friedländer's cocci in several respects, to be presently studied. Fraenkel further



investigated a few cases of pneumonia, and isolated from them cocci identical in microscopic appearances, cultures, and pathogenic effects, with those isolated in sputum septicæmia. The most extensive investigations on the whole question were those of Weichselbaum, published in 1886. This author examined 129 cases of the disease, including cases not only of acute croupous pneumonia, but of lobular and septic pneumonia. From them he isolated four groups of organisms. (1) *Diplococcus pneumoniae*. This he described as an oval or lancet-formed coccus, corresponding in appearance and growth characters to Fraenkel's coccus. (2) *Streptococcus pneumoniae*. This on the whole presented similar characters to the last, but it was more vigorous in its growth, and could grow below 20° C., though it preferred a temperature of 37° C. (3) *Staphylococcus pyogenus aureus*. (4) *Bacillus pneumoniae*. This was a rod-shaped organism, and was identical with Friedländer's pneumococcus. Of these organisms the diplococcus pneumoniae was by far the most frequent. It also occurred in all forms of pneumonia. Next in frequency was the streptococcus pneumoniae, and lastly the bacillus pneumoniae. Inoculation experiments were also performed by Weichselbaum with each of the three characteristic cocci he isolated. The diplococcus pneumoniae and the streptococcus pneumoniae both gave pathogenic effects of a similar kind in certain animals.

The general result of these earlier observations was to establish the occurrence in connection with pneumonia of two species of organisms, each having its distinctive characters, viz:—

1. *Fraenkel's pneumococcus*, which is recognised to be identical with the coccus of "sputum septicæmia," with Weichselbaum's diplococcus pneumoniae, and with his streptococcus pneumoniae.

2. *Friedländer's pneumococcus* (now known as Friedländer's pneumobacillus), which is almost certainly the bacillus pneumoniae of Weichselbaum.

We shall use the terms "*Fraenkel's pneumococcus*" and "*Friedländer's pneumobacillus*," as these are now usually applied to the two organisms.

#### **Microscopic Characters of the Bacteria of Pneumonia.—**

*Methods.*—The organisms present in acute pneumonia can best be examined in film preparations made from pneumonic lung (preferably from a part in a stage of acute congestion or early hepatisation), or from the gelatinous parts of pneumonic sputum (here again preferably when such sputum is either rusty or occurs early in the disease), or in sections of pneumonic lung. Such preparations may be stained by any of the ordinary weak stains, such as a watery solution of methylene-blue, but Gram's method is to be preferred, with Bismarck-brown or Ziehl-Neelsen carbol-fuchsin (one part to ten of water) as a contrast stain; with the latter it is best either to stain for only a few seconds, or to overstain and then decolorise with alcohol till the ground of the preparation is just tinted. The capsules can also be

stained by the methods already described (p. 102). In such preparations as the above, and even in specimens taken from the lungs immediately after death (as may be quite well done by means of a hypodermic syringe), putrefactive and other bacteria may be present, but those to be looked for are capsulated organisms, which may be of either or both of the varieties mentioned.

(1) *Fraenkel's Pneumococcus*.—This organism occurs in the form of a small oval coccus, about  $1\ \mu$  in longest diameter, arranged generally in pairs (diplococci), but also in chains of four to ten (Fig. 64). The free ends are often pointed like a lancet, hence the term *diplococcus lanceolatus* has also been applied to it. These cocci have round them a capsule, which, in films stained by ordinary methods, usually appears as an unstained halo, but is sometimes stained more deeply than the ground of the preparation. This difference in staining depends, in part at least, on the amount of decolorisation to which the preparation has been subjected. The capsule is rather broader than the body of the coccus, and has a sharply defined external margin. This organism takes up the basic aniline stains with great readiness, and also *retains the stain in Gram's method*. It is the organism of by far the most frequent occurrence in true croupous pneumonia, and in fact may be said to be rarely absent.



FIG. 64.—Film preparation of pneumonic sputum, showing numerous pneumococci (Fraenkel's) with unstained capsules; some are arranged in short chains. Stained with carbol-fuchsin.  $\times 1000$ .

(2) *Friedländer's Pneumobacillus*.—As seen in the sputum and tissues, this organism, both in its appearance and arrangement, as also in the presence of a capsule, somewhat resembles Fraenkel's pneumococcus, and it was at first described as the "pneumococcus." The form, however, is more of a short rod-shape, and it has blunt rounded ends; it is also rather broader than Fraenkel's pneumococcus. It is now classed amongst the bacilli, especially in view of the fact that elongated

rod forms may occur (Fig. 65). The capsule has the same

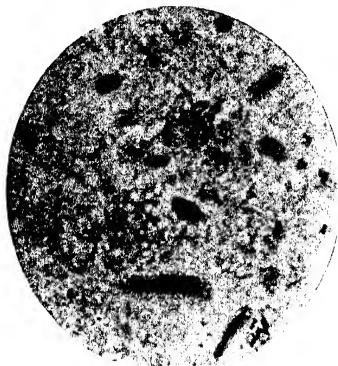


FIG. 65.—Friedländer's pneumobacillus, showing the variations in length, also capsules. Film preparation from exudate in a case of pneumonia.  $\times 1000$ .

monia than Fraenkel's; sometimes latter; very rarely it occurs alone.

In sputum preparations the capsule of both pneumococci may not be recognisable, and the same is sometimes true of lung preparations. This is probably due to changes which occur in the capsule as the result of changes in the vitality of the organisms. Sometimes in preparations stained by ordinary methods the difficulty of recognising the capsule when it is present, is due to the refractive index of the fluid in which the specimen is mounted being almost identical with that of the capsule. This

general characters as that of Fraenkel's organism. Friedländer's pneumobacillus stains readily with the basic aniline stains, but *loses the stain in Gram's method*, and is accordingly coloured with the contrast stain,--fuchsin or Bismarck-brown, as above recommended. A valuable means is thus afforded of distinguishing it from Fraenkel's pneumococcus in microscopic preparations.

Friedländer's organism is much less frequently present in pneumonia associated with the

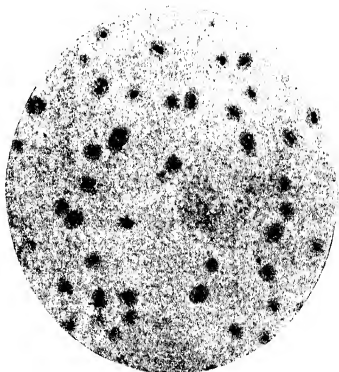


FIG. 66.—Fraenkel's pneumococcus in serous exudation at site of inoculation in a rabbit, showing capsules stained. Stained by Rd. Muir's method.  $\times 1000$ .

This

difficulty can always be overcome by having the groundwork of the preparation tinted.

**The Cultivation of Fraenkel's Pneumococcus.**—It is usually difficult, and sometimes impossible, to isolate this coccus directly from pneumonic sputum. On culture media it has not a vigorous growth, and when mixed with other bacteria it is apt to be overgrown by the latter. To get a pure culture it is best to insert a small piece of the sputum beneath the skin of a rabbit or a mouse. In about forty-eight hours the animal will die, with numerous capsulated pneumococci throughout its blood. From the heart-blood cultures can be easily obtained. Cultures can also be got *post mortem* from the lungs of pneumonic patients by streaking a number of agar or blood-agar tubes with a scraping taken from the area of acute congestion or commencing red hepatisation, and incubating them at 37° C. The colonies of the pneumococcus appear as almost transparent small discs which have been compared to drops of dew (Fig. 67). This method is also sometimes successful in the case of sputum.

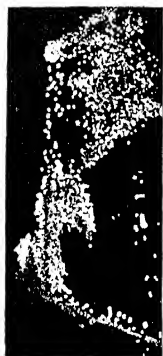


FIG. 67.—Stroke culture of Fraenkel's pneumococcus on blood agar. The colonies are large and unusually distinct. Twenty-four hours' growth at 37° C. Natural size.

The appearances presented in cultures by different varieties of the pneumococcus vary somewhat. It always grows best on blood serum or on Pfeiffer's blood agar. It usually grows well on ordinary agar or in bouillon, but not so well on glycerin agar. In a stroke culture on *blood serum* growth appears as an almost transparent pellicle along the track, with isolated colonies at the margin. On *agar* media it is more manifest, but otherwise has similar characters. The appearances are similar to those of a culture of streptococcus pyogenes, but the growth is less vigorous, and is more delicate in appearance. A similar statement also applies to cultures in *gelatin* at 22° C., growth in a stab culture appearing as a row of minute points which remain of small size; there is, of course, no liquefaction of the medium. On *agar* plates colonies are almost invisible to the naked eye, but under a low power of the microscope appear to have a compact finely granular centre and a pale transparent periphery. In *bouillon*, growth forms a slight turbidity, which settles to the

bottom of the vessel as a slight dust-like deposit. On *potatoes*, as a rule, no growth appears. Cultures on such media may be maintained for one or two months, if fresh sub-cultures are made every four or five days, but they tend ultimately to die out. They also rapidly lose their virulence, so that four or five days after isolation from an animal's body their pathogenic action is already diminished. Eyre and Washbourn, however, have succeeded in maintaining cultures in a condition of constant virulence for at least three months by growing the organisms on



FIG. 68.—Fraenkel's pneumococcus from a pure culture on blood agar of twenty-four hours' growth, some in pairs, some in short chains. Stained with weak carbol-fuchsin.  $\times 1000$ .

agar smeared with rabbits' blood. The agar must be prepared with Witte's peptone, must not be heated over  $100^{\circ}$  C., and after neutralisation (rosolic acid being used as the indicator) must have 5 per cent of normal sodium hydrate added. The tubes when inoculated are to be kept at  $37.5^{\circ}$  C. and sealed to prevent evaporation. In none of the ordinary artificial media do pneumococci develop a capsule. They usually appear as diplococci, but in preparations made from the surface of agar or from

bouillon, shorter or longer chains may be observed (Fig. 68). After a few days' growth they lose their regular shape and size, and involution forms appear. Usually the pneumococcus does not grow below  $22^{\circ}$  C., but forms in which the virulence has disappeared often grow well at  $20^{\circ}$  C. Its optimum temperature is  $37^{\circ}$  C., its maximum  $42^{\circ}$  C. It is preferably an aerobe, but can exist without oxygen. It prefers a slightly alkaline medium to a neutral, and does not grow on an acid medium. These facts show that when growing outside the body on artificial media, the pneumococcus is a comparatively delicate organism. There has been described by Eyre and Washbourn a non-pathogenic type of the pneumococcus which may be found in the healthy mouth, and which may also be produced during the saprophytic growth of the virulent form. From the latter it

differs generally in its more vigorous growth, in producing a uniform cloud in bouillon, in slowly liquefying gelatin, and in growing on potato.

**The Cultivation of Friedländer's Pneumobacillus.**—This organism, when present in sputum or in a pneumonic lung, can be readily separated by making ordinary gelatin plate cultures, or a series of successive strokes on agar tubes. The surface colonies always appear as white discs which become raised from the surface so as to appear like little knobs of ivory. From these, pure cultures can be readily obtained. The appearance of a stab culture in gelatin growth is very characteristic. At the site of the puncture, there is on the surface a white growth heaped up, it may be fully one-eighth of an inch above the level of the gelatin; along the needle track there is a white



FIG. 69.—Stab culture of Friedländer's pneumobacillus in peptone gelatin, showing the nail-like appearance; ten days' growth. Natural size.



FIG. 70.—Friedländer's pneumobacillus,<sup>1</sup> from a young culture on agar, showing some rod-shaped forms. Stained with thionin-blue.  $\times 1000$ .

granular appearance, so that the whole resembles a white round-headed nail driven into the gelatin (Fig. 69). Hence the name

<sup>1</sup> The apparent size of this organism, on account of the nature of its sheath, varies much according to the stain used. If stained with a strong stain, *e.g.* carbol-fuchsin, its thickness appears nearly twice as great as is shown in the figure.

“nail-like” which has been applied. Occasionally bubbles of gas develop along the line of growth. There is no liquefaction of the medium. On sloped *agar* it forms a very white growth with a shiny lustre, which, when touched with a platinum needle, is found to be of a viscous consistence. In cultures much longer rods are formed than in the tissues of the body (Fig. 70). On the surface of *potatoes* it forms an abundant moist white layer. Friedländer's bacillus has active fermenting powers on sugars, though varieties isolated by different observers vary in the degree in which such powers are possessed. It always seems capable of acting on dextrose, lactose, maltose, dextrin, and mannite, and sometimes also on glycerin. The substances produced by the fermentation vary with the sugar fermented, but include ethylic alcohol, acetic acid, levolactic acid, succinic acid, along with hydrogen and carbonic acid gas. The amount of acid produced from lactose seems only exceptionally sufficient to cause coagulation of milk. It is said by some that this bacillus is identical with an organism common in sour milk, and also a normal inhabitant of the human intestine, viz. the bacterium *lactis aerogenes* of Escherich.

**The Occurrence of the Pneumobacteria in Pneumonia and other Conditions.**—Capsulated organisms have been found in every variety of the disease—in acute croupous pneumonia, in broncho-pneumonia, in septic pneumonia. In the great majority of these it is Fraenkel's pneumococcus which both microscopically and culturally has been found to be present. Friedländer's pneumobacillus occurs in only about 5 per cent of the cases. It may be present alone or associated with Fraenkel's organism. In a case of croupous pneumonia the pneumococci are found all through the affected area in the lung, especially in the exudation in the air-cells. They also occur in the pleural exudation and effusion, and in the lymphatics of the lung. The greatest number are found in the parts where the inflammatory process is most recent, *e.g.* in an area of acute congestion in a case of croupous pneumonia, and therefore such parts are preferably to be selected for microscopic examination, and as the source of cultures. Sometimes there occur in pneumonic consolidation areas of suppurative softening, which may spread diffusely. In such areas the pneumococci occur with or without ordinary pyogenic organisms, streptococci being the commonest concomitants. In other cases, especially when the condition is secondary to influenza, gangrene may supervene and lead to destruction of large portions of the lung. In these a great variety of bacteria, both aerobes and anaerobes, are to be found.

In ordinary broncho-pneumonias also Fraenkel's pneumococcus is usually present, sometimes along with pyogenic cocci; in the broncho-pneumonias secondary to diphtheria it may be accompanied by the diphtheria bacillus, and also by pyogenic cocci; in typhoid pneumonias the typhoid bacillus or the *b. coli* may be alone present or be accompanied by the pneumococcus, and in influenza pneumonias the influenza bacillus may occur. In septic pneumonias the pyogenic cocci in many cases are the only organisms discoverable, but the pneumococcus may also be present. Especially important, as we shall see, from the point of view of the etiology of the disease, is the occurrence in other parts of the body of pathological conditions associated with the presence of the pneumococcus. By direct extension to neighbouring parts empyema, pericarditis, and lymphatic enlargements in the mediastinum and neck may take place; in the first the pneumococcus may occur either alone or with pyogenic cocci. But distant parts may be affected, and the pneumococcus may be found in suppurations and inflammations in various parts of the body (subcutaneous tissue, peritoneum, joints, kidneys, liver, etc.), in otitis media, ulcerative endocarditis (p. 188), and meningitis. These conditions may take place either as complications of pneumonia, or they may constitute the primary disease. The occurrence of meningitis is of special importance, for next to the lungs the meninges appear to be the parts most liable to attack by the pneumococcus. A large number of cases have been investigated by Netter, who gives the following tables of the relative frequency of the primary infections by the pneumococcus in man:—

(1) In adults—

|                         |                |
|-------------------------|----------------|
| Pneumonia . . . . .     | 65·95 per cent |
| Broncho-pneumonia )     |                |
| Capillary bronchitis )  | 15·85 „        |
| Meningitis . . . . .    | 13·00 „        |
| Empyema . . . . .       | 8·53 „         |
| Otitis . . . . .        | 2·44 „         |
| Endocarditis . . . . .  | 1·22 „         |
| Liver abscess . . . . . | 1·22 „         |

(2) In children 46 cases were investigated. In 29 the primary affection was otitis media, in 12 broncho-pneumonia, in 2 meningitis, in 1 pneumonia, in 1 pleurisy, in 1 pericarditis.

Thus in children the primary source of infection is in a great many cases an otitis media, and Netter concludes that infection takes place in such conditions from the nasal cavities.

As bearing on the occurrence of pneumococcal infections



secondary to such a local lesion as pneumonia, it is important to note that in a large proportion of cases of the latter disease the pneumococcus can be isolated from the blood.

**Experimental Inoculation.**—The *pneumococcus* of Fraenkel is pathogenic to various animals, though the effects vary somewhat with the virulence of the race used. The susceptibility of different species, as Gamaleia has shown, varies to a considerable

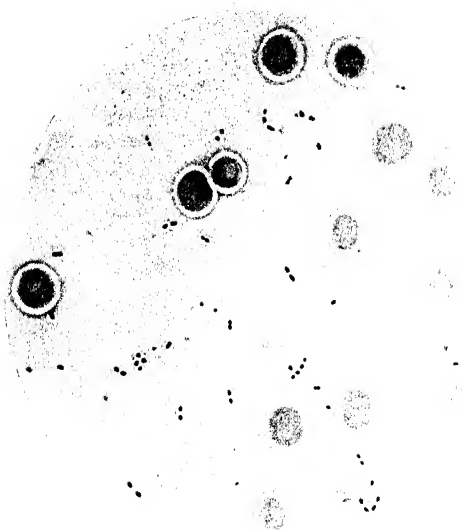


FIG. 71.—Capsulated pneumococci in blood taken from the heart of a rabbit, dead after inoculation with pneumonic sputum.

Dried film, fixed with corrosive sublimate. Stained with carbol-fuchsin and partly decolorised.  $\times 1000$ .

extent. The rabbit, and especially the mouse, are very susceptible; the guinea-pig, the rat, the dog, and the sheep occupy an intermediate position; the pigeon is immune. In the more susceptible animals the general type of the disease produced is not pneumonia, but a general *septicæmia*. Thus, if a rabbit or a mouse be injected subcutaneously with pneumonic sputum, or with a scraping from a pneumonic lung, death occurs in from twenty-four to forty-eight hours. There is some fibrinous infiltration at the point of inoculation, the spleen is

often enlarged and firm, and the blood contains capsulated pneumococci in large numbers (Fig. 71). If the seat of inoculation be in the lung, there generally results pleuritic effusion on both sides, and in the lung there may be a process somewhat resembling the early stage of acute croupous pneumonia in man. There are often also pericarditis and enlargement of spleen. We have already stated that cultures of the pneumococci on artificial media in a few days begin to lose their virulence. Now, if such a partly attenuated culture be injected subcutaneously into a rabbit, there is greater local reaction; pneumonia, with exudation of lymph on the surface of the pleura, and a similar condition in the peritoneum, may occur. In sheep greater immunity is marked by the occurrence, after subcutaneous inoculation, of an enormous local sero-fibrinous exudation, and by the fact that few pneumococci are found in the blood stream. Intra-pulmonary injection in sheep is followed by a typical pneumonia, which is generally fatal. The dog is still more immune; in it also intra-pulmonary injection is followed by a fibrinous pneumonia, which is only sometimes fatal. Inoculation by inhalation appears only to have been performed in the susceptible mouse and rabbit; here also septicæmia resulted.

The general conclusion to be drawn from these experiments thus is that in highly susceptible animals virulent pneumococci produce a general septicæmia; whereas in more immune species there is an acute local reaction at the point of inoculation, and if the latter be in the lung, then there may result pneumonia, which, of course, is merely a local acute inflammation occurring in a special tissue, but identical in essential pathology with an inflammatory reaction in any other part of the body. When a dose of pneumococci sufficient to kill a rabbit is injected subcutaneously in the human subject, it gives rise to a local inflammatory swelling with redness and slight rise of temperature, all of which pass off in a few days. It is therefore justifiable to suppose that man occupies an intermediate place in the scale of susceptibility, probably between the dog and the sheep, and that when the pneumococcus gains an entrance to his lungs, the local reaction in the form of pneumonia occurs. In this connection the occurrence of manifestations of general infection associated with pneumonia in man is of the highest importance. We have seen that meningitis and other inflammations are not very rare complications of the disease, and such cases form a link connecting the local disease in the human subject with the general septicæmic processes which may be produced

artificially in the more susceptible representatives of the lower animals.

A fact which at first appeared rather to militate against the pneumococcus being the cause of pneumonia was the discovery of this organism in the saliva of healthy men. This fact was early pointed out by Pasteur, and also by Fraenkel, and the observation has been confirmed by many other observers. It can certainly be isolated from the mouths of a considerable proportion of normal men, from their nasal cavities, etc., being probably in any particular individual more numerous at some times than at others, and sometimes being entirely absent. This can be proved, of course, by inoculation of susceptible animals. Such a fact, however, only indicates the importance of predisposing causes in the etiology of the disease, and it is further to be observed that we have corresponding facts in the case of the diseases caused by pyogenic staphylococci, streptococci, the bacillus coli, etc. It is probable that by various causes the vitality and power of resistance of the lung are diminished, and that then the pneumococcus gains an entrance. In relation to this possibility we have the very striking facts that in the irregular forms of pneumonia, secondary to such conditions as typhoid and diphtheria, the pneumococcus is very frequently present, alone or with other organisms. Apparently the effects produced by such bacteria as the *b. typhosus* and the *b. diphtheriae* can devitalise the lung to such an extent that secondary infection by the pneumococcus is more likely to occur and set up pneumonia. We can therefore understand how much less definite devitalising agents such as cold, alcoholic excess, etc., can play an important part in the causation of pneumonia. In this way also other abnormal conditions of the respiratory tract, a slight bronchitis, etc., may play a similar part.

It is more difficult to explain why sometimes the pneumococcus is associated with a spreading inflammation, as in croupous pneumonia, whilst at other times it is localised to the catarrhal patches in broncho-pneumonia. It is quite likely that in the former condition the organism is possessed of a different order of virulence, though of this we have no direct proof. We have, however, a closely analogous fact in the case of erysipelas; this disease, we have stated reasons for believing, is produced by a streptococcus which, when less virulent, causes only local inflammatory and suppurative conditions.

*Summary.*—We may accordingly summarise the facts regarding the relation of Fraenkel's pneumococcus to the disease

by saying that it can be isolated from nearly all cases of acute croupous pneumonia, and also from a considerable proportion of other forms of pneumonia. When injected into the lungs of moderately insusceptible animals it gives rise to pneumonia. If, in default of the crucial experiment of intra-pulmonary injection in the human subject, we take into account the facts we have discussed, we are justified in holding that it is the chief factor in causing croupous pneumonia, and also plays an important part in other forms. Pneumonia, in the widest sense of the term, is, however, not a specific affection, and various inflammatory conditions in the lungs can be set up by the different pyogenic organisms, by the bacilli of diphtheria, of influenza, etc.

The possibility of Friedländer's *pneumobacillus* having an etiological relationship to pneumonia has been much disputed. Its discoverer found that it was pathogenic towards mice and guinea-pigs, and to a less extent towards dogs. Rabbits appeared to be immune. The type of the disease was of the nature of a septicæmia. No extended experiments, such as those performed by Gamaleia with Fraenkel's coccus, have been done, and therefore we cannot say whether any similar pneumonic effects are produced by it in partly susceptible animals. The organism appears to be present alone in a small number of cases of pneumonia, and the fact that it also appears to have been the only organism present in certain septicæmic complications of pneumonia, such as empyema and meningitis, render it possible that it may be the causal agent in a few cases of the disease.

In the septic pneumonias the different pyogenic organisms already described are found, and sometimes in ordinary pneumonias, especially the catarrhal forms, other organisms, such as the *b. coli* or its allies, may be the causal agents.

**The Pathology of Pneumococcus Infection.**—The effects of the action of the pneumococcus, at any rate in a relatively insusceptible animal such as man, seem to indicate that toxins must play an important part. Pneumonia is a disease which presents in many respects the characters of an acute poisoning. In very few cases does death take place from the functions of the lungs being interfered with to such an extent as to cause asphyxia. It is from cardiac failure, from grave interference with the heat-regulating mechanism, and from general nervous depression that death usually results. These considerations, taken in connection with the fact that in man the organisms are found in the greatest numbers in the lung, suggest that a toxic action is at work. Various attempts have been made to isolate toxins from cultures of the pneumococcus, *e.g.* by precipitating

bouillon cultures with alcohol or ammonium sulphate, and poisonous effects have been produced by certain substances thus derived; but the effects produced are, as in so many other similar cases, of a non-specific character and to be classed as interferences with general metabolism. The general conclusion has been that the toxins at work in pneumonia are intracellular; but no special light has been thrown on the common effects of the members of this group of bacterial poisons.

*Immunisation against the Pneumococcus.*—Animals can be immunised against the pneumococcus by inoculation with cultures which have become attenuated by growth on artificial media, or with the naturally attenuated cocci which occur in the sputum after the crisis of the disease. Netter effected immunisation by injecting an emulsion of the dried spleen of an animal dead of pneumococcus septicæmia. Virulent cultures killed by heating at 62° C., rusty sputum kept at 60° C. for one to two hours and then filtered, and filtered or unfiltered bouillon cultures similarly treated have also been used. In all cases one or two injections, at intervals of several days, are sufficient for immunisation, but the immunity has often been observed to be of a fleeting character and may not last more than a few weeks. The serum of such immunised animals protects rabbits against subsequent inoculation with pneumococci, and if injected within twenty-four hours after inoculation, prevents death. A protective serum was obtained by Washbourn, who employed pneumococcus cultures of constant virulence. This observer immunised a pony by using successively (1) broth cultures killed by one hour's exposure to 60° C.; (2) living agar cultures; (3) living broth cultures. From this animal there was obtained a serum which protected susceptible animals against many times an otherwise fatal dose, and which also had a limited curative action. It is stated that the serum of patients who have recovered from pneumonia has in a certain proportion of cases a protective effect against the pneumococcus in rabbits, similar to that exhibited by the serum of immune animals.

The Klemperers treated a certain number of cases of human pneumonia by serum derived from immune animals, and apparently with a certain measure of success, and sera prepared by Washbourn and by others have also been used. The results obtained by different observers have, however, been rather contradictory. The use of these sera apparently causes the temperature in some cases to fall, and even may hasten a crisis, but further experience is necessary before their value in therapeutics can be properly estimated.

There has been considerable difference of opinion as to the explanations to be given of the facts observed regarding immunisation against the pneumococcus and especially regarding the properties of immune sera. At first these sera were supposed to possess antitoxic qualities—largely on the ground that no bactericidal effect was produced by them on the bacterium in vitro. As no specific toxin has been proved to be concerned in the action of the organism the development of an antitoxin during immunisation must, in the present state of knowledge, be looked on as not yet proved. To explain the action of a serum in preventing and curing pneumococcal infections, it has been thought to have the complex character seen in antityphoid sera in which two substances—immune body and complement (see Immunity)—are concerned, and the variability in the therapeutic results obtained has been accounted for on the view that there might be a deficiency of complement, such as occurs in other similar cases. The absence of bactericidal effect, however, raises several difficult points. It is stated that no such effect is observable either in immune sera, or in the serum of patients who have successfully come through an attack of the natural disease. Some effect of the kind would be expected to be present if the anti-pneumonic serum were quite comparable to the antityphoid serum. Within recent times many have turned to the opsonic property of sera to account for the facts observed. In this connection Mennes observed that normal leucocytes only become phagocytic towards pneumococci when they are lying in the serum of an animal immunised against this bacterium. Wright had in his early papers looked to the phagocytosis of sensitised bacteria to explain their destruction in the absence of bactericidal qualities in the serum alone, and Neufeld and Rimpan have described the occurrence of an opsonic effect in the action of an anti-pneumococcal serum. Further work may show that along these lines lies the explanation of the facts observed.

In studying further the relationship of the opsonic effect to pneumococcal infection, inquiry has been directed to the opsonic qualities of the blood of pneumonic patients, especially with a view to throwing light on the nature of the febrile crisis. According to some results the opsonic index as compared with that of a healthy person is not above normal, but if the possible phagocytic capacities of the whole blood of the sick person be taken into account these will probably be much above normal in consequence of the leucocytosis which usually accompanies a successful resistance to this infection. It has been observed,

however, that as the crisis approaches in a case which is to recover the opsonic index rises, and after defervescence gradually falls to normal. And further, as bearing on the factors involved in the successful resistance of the organism to the pneumococcus, it has been noted that avirulent pneumococci are more readily opsonised than more virulent strains. Further observations along such lines are to be looked for with interest, and it may be said that Wright's vaccination methods have been applied to the treatment of pneumonia cases, and in certain instances are said to have been followed by favourable result. It may be noted here, in conclusion, that in man it is probable that immunity against pneumonia may be short-lived, as in a good many cases of pneumonia a history of a previous attack is elicited.

*Agglutination of the Pneumococcus.*—If a small amount of a culture of Fraenkel's pneumococcus be placed in an anti-pneumococcic serum, an aggregation of the bacteria into clumps occurs. Such an agglutination, as it is called, is frequently observed under similar circumstances with other bacteria. The phenomenon is not invariably associated with the presence of protective bodies in a serum, but it has been used for diagnostic purposes in the differentiation of sore throats due to pneumococcus infection from those due to other bacteria. Whether the method is reliable has still to be proved.

**Methods of Examination.**—These have been already described, but may be summarised thus: (1) Microscopic. Stain films from the densest part of the sputum or from the area of spreading inflammation in the lung by Gram's method and by carbol-fuchsin, etc. (pp. 99, 101), in the latter case without decolorising the groundwork of the preparation.

(2) By cultures. (a) *Fraenkel's pneumococcus*. With similar material make successive strokes on agar, blood agar, or blood serum. The most certain method, however, is to inject some of the material containing the suspected cocci into a rabbit. If the pneumococcus be present the animal will die, usually within forty-eight hours, with numerous capsulated pneumococci in its heart blood. With the latter inoculate tubes of the above media and observe the growth. In some cases of severe pneumococcic infection the organism may be cultivated from the blood obtained by venesection (p. 68). (b) *Friedländer's pneumobacillus* can be readily isolated either by ordinary gelatin plates or by successive strokes on agar media.

## EPIDEMIC CEREBRO-SPINAL MENINGITIS.

As the result of observations on this disease in different parts of the world, it has been now established that the causal agent is the *diplococcus intracellularis meningitidis* first described by Weichselbaum. This organism is a small coccus measuring about  $1\ \mu$  in diameter and usually occurs in pairs, the adjacent sides being somewhat flattened against each other. In most cases the cocci are chiefly contained within polymorphonuclear leucocytes in the exudation (Fig. 72); in some cases, however, the majority may be lying free. It stains readily with basic aniline dyes, but loses the stain in Gram's method, the readiness with which the organism decolorises varying with different strains. Both in appearance and in its staining reactions it is superficially similar to the gonococcus (*vide infra*). The organism can readily be cultivated outside the body, but the conditions of growth are somewhat restricted—agar with an admixture of serum or blood (preferably human) is most suitable.<sup>1</sup> Strains



FIG. 72.—Film preparation of exudation from a case of meningitis, showing the diplococci within leucocytes. Stained with carbol-thionin-blue.  $\times 1000$ .

separated in different epidemics appear to present slight individual variations, but the following description may be taken as summing up the common characters. Growth takes place best at the temperature of the body, and practically ceases at  $25^{\circ}\text{C}$ . On serum agar the colonies are circular discs of almost transparent appearance and possessing a smooth shining surface; they have little tendency to become confluent. When examined under a low magnification the colour is seen to be somewhat yellowish and the margins usually are smooth and

<sup>1</sup> A very good medium is one composed of 1 part of ascitic fluid and 6 parts of 1 per cent glucose agar; the serum obtained aseptically is added to the agar in the melted state at  $45^{\circ}\text{C}$ . and the tubes are tested as regards sterility by incubation.



canal marked congestion and inflammatory change in the nasal mucous membrane followed, and in this position he was able to find a Gram-negative diplococcus; he was, however, unable to recover the diplococcus intracellularis in culture from this situation. These results would seem to indicate that the organism might spread from the brain to the nasal cavity, but if this be so, it also follows that an extension may take place in the reverse direction. On the whole the evidence at present tends to show that the entrance of the organism into the body is by the naso-pharynx, and that this usually results by inhalation of the organism distributed in fine particles of expectoration, etc. In fact, as regards the mode and conditions of infection, an analogy would appear to hold between this disease and influenza.

Apart from the epidemic form of the disease, cases of sporadic nature also occur, in which the lesions are of the same nature, and in which the diplococcus intracellularis is present. The facts stated would indicate that the origin and spread of the disease in the epidemic form depend on certain conditions which produce an increased virulence of the organism. We are, however, as yet entirely ignorant as to what these conditions may be. In simple posterior basal meningitis in children, a diplococcus is present, as described by Still, which has the same microscopic and cultural characters as the diplococcus intracellularis; it has been regarded as probably an attenuated variety of the latter. Recently, however, Houston and Rankin have found that the serum of a patient suffering from epidemic meningitis does not exert the same opsonic and agglutinative effects on the diplococcus of basal meningitis as on the diplococcus intracellularis; and this result points to the two organisms being distinct, though closely allied, species.

An agglutination reaction towards the diplococcus intracellularis is given by the serum of patients suffering from the disease, where life is prolonged for a sufficient length of time, but the degree of the reaction does not possess much clinical significance. It usually appears about the fourth day, when the serum may give a positive reaction in a dilution of 1:50; at a later stage it has been observed in so great a dilution as 1:1000. There is thus no doubt that anti-substances are produced in epidemic meningitis as in other diseases, and this is also found to be the case on inoculation of animals with pure cultures. Attempts had been made to obtain an anti-serum, and a certain measure of success has been obtained so far as experimental results are concerned. Flexner obtained such a serum from a goat, and

found that it had a certain protective effect in guinea-pigs and monkeys against infection by the organism, but, on the whole, better results were obtained with the serum of inoculated monkeys. As yet no important applications towards the treatment of the disease have been effected.

In the nasal cavity there occur other diplococci which have a close resemblance to the *diplococcus intracellularis*. These occur in the healthy state but are especially abundant in catarrhal conditions; of these the *diplococcus catarrhalis* has the closest resemblance to the *diplococcus intracellularis*. In addition to occurring in health this organism has also been found in large numbers in epidemic catarrh. Its microscopic appearances are practically similar to those described above, and it also occurs within leucocytes. Its colonies on serum agar are more opaque than those of the *diplococcus intracellularis*, and they have a tough consistence, so that they are sometimes removed *en masse* by the platinum needle. The organism grows on gelatin at 20° C. without liquefying the medium, and it has none of the fermentative properties described above as belonging to the *diplococcus intracellularis*. Other species of Gram-negative micrococci have also been isolated, and a Gram-positive diplococcus called the *diplococcus crassus* is of common occurrence: this organism is rather larger than the *diplococcus intracellularis*, and especially in sub-cultures may tend to assume staphylococcal forms. It is thus evident that the nasal cavity is the common habitat for a number of closely allied diplococci, and that the identification of any suspected organism as the *diplococcus intracellularis* can only be effected by cultivation tests.

Apart from the epidemic form of the disease, meningitis may be produced by almost any of the organisms described in the previous chapter, as associated with inflammatory conditions. A considerable number of cases, especially in children, are due to the pneumococcus. In many instances where no other lesions are present the extension is by the Eustachian tube to the middle ear. In other cases the path of infection is from some other lesion by means of the blood stream. This organism also infects the meninges not infrequently in lobar pneumonia, and in some cases with head symptoms we have found it present where there was merely a condition of congestion. The pneumobacillus also has been found in a few cases. Meningitis is not infrequently produced by streptococci, especially when middle ear disease is present, less frequently by one of the staphylococci; occasionally more than one organism may be concerned. In meningitis following influenza the influenza bacillus has been found in a

cells, but when it becomes purulent the large proportion within leucocytes is a very striking feature. In the leucocytes they lie within the protoplasm, especially superficially, and are often so



FIG. 74.—Portion of film of gonorrhoeal pus, showing the characteristic arrangement of the gonococci within leucocytes. Stained with fuchsin.  $\times 1000$ .

numerous that the leucocytes appear to be filled with them, and their nuclei are obscured. As the disease becomes more chronic, the gonococci gradually become diminished in number, though even in long-standing cases they may still be found in considerable numbers. They are also present in the purulent secretion of gonorrhoeal conjunctivitis, also in various parts of the female genital organs when these parts are the seat of true gonorrhoeal infection, and they have been found in some cases in the second-

ary infections of the joints in the disease, as will be described below.

**Staining.**—The gonococcus stains readily and deeply with a watery solution of any of the basic aniline dyes—methylene-blue, fuchsin, etc. It is, however, easily decolorised, and it completely loses the stain by Gram's method—an important point in the microscopical examination.

**Cultivation of the Gonococcus.**—This is attended with some difficulty, as the suitable media and conditions of growth are somewhat restricted. The most suitable media are solidified blood serum (especially human serum and rabbit's serum), "blood agar," and Wertheim's medium, which consists of one part of fluid serum added to two parts of liquefied agar at a temperature of  $40^{\circ}\text{C}$ . and then allowed to solidify by cooling. The serum may be obtained from the blood of the human placenta; pleuritic or other effusion may also be used. Growth takes place best at the temperature of the body, and ceases altogether at  $25^{\circ}\text{C}$ . Cultures are obtained by taking some pus on the loop of the platinum needle and inoculating one of the media mentioned by leaving minute quantities here and there on the surface. The medium may be used either as

ordinary "sloped tubes" or as a thin layer in a Petri's capsule. The young colonies are visible within forty-eight hours, and often within twenty-four hours. They appear around the points of inoculation as small semi-transparent discs of irregularly rounded shape, the margin being undulated and sometimes showing small processes. The colonies vary somewhat in size and tend to remain more or less separate. They generally reach their maximum size on the fourth or fifth day, and are usually found to be dead on the ninth day, sometimes earlier. On the medium of Wertheim the period of active growth and the duration of life are somewhat longer. Even if impurities are present, pure sub-cultures can generally be obtained by the above method from colonies of the gonococcus which may be lying separate. In the early stage of the disease the organism is present in the male urethra in practically pure condition, and if the meatus of the urethra be sterilised by washing with weak solution of corrosive sublimate and then with absolute alcohol, and the material for inoculation be expressed from the deeper part of the urethra, cultures may often be obtained which are pure from the first. By successive sub-cultures at short intervals, growth may be maintained indefinitely, and the organism gradually flourishes more luxuriantly. In culture the organisms have similar microscopic characters to those described (Fig. 75), but show a remarkable tendency to undergo degeneration, becoming swollen and of various sizes, and staining very irregularly. Degenerated forms are seen even on the second day, whilst in a culture four or five days old comparatively few normal cocci may be found. The less suitable the medium the more rapidly does degeneration take place.



FIG. 75. Gonococci, from a pure culture on blood agar of twenty-four hours' growth. Some already are beginning to show the swollen appearance common in older cultures. Stained with carbol-thionin-blue.  $\times 1000$ .

On ordinary agar and on glycerin agar growth does not take place, or is so slight that these media are quite unsuitable for

purposes of culture. The organism does not grow on gelatin,<sup>1</sup> potato, etc.

*Plate-Cultures.*—The following ingenious method of plate-culture was introduced by Wertheim for the culture of the gonococcus. The medium of culture is a mixture of human blood serum and of ordinary agar (2 per cent) in equal parts. The serum, in a fluid and sterile condition, is put in suitable quantities into two or three test tubes and brought to a temperature of 40° C. These are then successively inoculated with the pus or other material in the same manner as gelatin tubes for ordinary plates (*vide* p. 52). To each tube is added an equal part of ordinary agar which has been thoroughly liquefied by heating and allowed to cool also to 40° C. The mixture is then thoroughly shaken up and quickly poured out on a plate or Petri's dish and allowed to solidify, the plates being then incubated at a temperature of 37° C. The colonies of the gonococcus are just visible in twenty-four hours, and are seen both in the substance of the medium and on the surface. The deep colonies when examined with a lens are minute and slightly nodulated spheres, sometimes showing little processes, whilst those on the surface are thin discs of larger diameter with wavy margin and rather darker centre. In this way the gonococcus may be separated from fluids which are contaminated with a considerable number of other organisms.

**Relations to the Disease.**—The gonococcus is invariably present in the urethral discharge in gonorrhoea, and also in other parts of the genital tract when these are the seat of true gonorrhoeal infection. Its presence in these different positions has been demonstrated not only by microscopic examination but also by culture. From the description of the conditions of growth in culture, it will be seen that a life outside the body in natural conditions is practically impossible—a statement which corresponds with the clinical fact that the disease is always transmitted directly by contagion. Inoculations of pure cultures on the urethra of lower animals, and even of apes, is followed by no effect, but a similar statement can be made with regard to inoculations of gonorrhoeal pus itself. In fact, hitherto it has been found impossible to reproduce the disease by any means in the lower animals. On a considerable number of occasions inoculations of pure cultures have been made on the human urethra, both in the male and female, and the disease, with all its characteristic symptoms, has resulted. (Such experiments have been performed independently by Bumm, Steinschneider, Wertheim, and others.) The causal relationship of the organism to the disease has therefore been completely

<sup>1</sup> Turro has announced that he has cultivated the gonococcus on acid gelatin, *i.e.* ordinary peptone gelatin which has not been neutralised. We have failed to obtain any growth of the gonococcus on this medium, even when inoculation was made from a vigorous growth on blood agar.

established, and it is interesting to note how the conditions of growth and the pathogenic effects of the organism agree with the characters of the natural disease.

Intraperitoneal injections of pure cultures of the gonococcus in white mice produce a localised peritonitis with a small amount of suppuration, the organisms being found in large numbers in the leucocytes (Wertheim). They also penetrate the peritoneal lining and are found in the sub-endothelial connective tissue, but they appear to have little power of proliferation, they soon disappear, and the inflammatory condition does not spread. Injection of pure cultures into the joints of rabbits, dogs, and guinea-pigs causes an acute inflammation, which, however, soon subsides, whilst the gonococci rapidly die out; a practically similar result is obtained when dead cultures are used. These experiments show that while the organism, when present in large numbers, can produce a certain amount of inflammatory change in these animals, it has little or no power of multiplying and spreading in their tissues.

**Toxin of the Gonococcus.**—De Christmas cultivated the gonococci in a mixture of one part of ascitic fluid and three parts of bouillon, and has found that the fluid after twelve days' growth has toxic properties. At this period all the organisms are dead; such a fluid constitutes the "toxin." The toxic substances are precipitated along with the proteids by alcohol, and the precipitate after being desiccated possesses the toxic action. In young rabbits injection of the toxin produces suppuration; this is well seen in the anterior chamber of the eye, where hypopyon results. The most interesting point, however, is with regard to its action on mucous surfaces; for, while in the case of animals it produces no effect, its introduction into the human urethra causes acute catarrh, attended with purulent discharge. He found that no tolerance to the toxin resulted after five successive injections at intervals. In a more recent publication he points out that the toxin on intracerebral injection has marked effects; he also claims to have produced an antitoxin. He states that the toxin diffuses out in the culture medium, and does not merely result from disintegration of the organisms. This has, however, been called in question by other investigators.

**Distribution in the Tissues.**—The gonococcus having been thus shown to be the direct cause of the disease, some additional facts may be given regarding its presence both in the primary and secondary lesions. In the human urethra the gonococci penetrate the mucous membrane, passing chiefly between the epithelial cells, causing a loosening and desquamation of many of the latter and inflammatory reaction in the tissues below, attended with great increase of secretion. There occurs also a gradually increasing emigration of leucocytes, which take up a large number of the organisms. The organisms also penetrate the subjacent connective tissue, and are especially found along with extensive leucocytic emigration around the lacunae. Here also many are contained within leucocytes. Even, however, when the gonococci have disappeared from the urethral discharge, they may still be present in the deeper part of the

mucous membrane of the urethra, possibly also in the prostate, and may thus be capable of producing infection. The prostatic secretion may sometimes be examined by making pressure on the prostate from the rectum when the patient has almost emptied his bladder, the secretion being afterwards discharged along with the remaining urine. (Foulerton.) In acute gonorrhœa there is often a considerable degree of inflammatory affection of the prostate and vesiculae seminales, but whether these conditions are always due to the presence of gonococci in the affected parts we have not at present the data for determining. A similar statement also applies to the occurrence of orchitis and also of cystitis in the early stage of gonorrhœa. Gonococci have, however, been obtained in pure culture from peri-urethral abscess and from epididymitis: it is likely that the latter condition, when occurring in gonorrhœa, is usually due to the actual presence of gonococci. During the more chronic stages other organisms may appear in the urethra, aid in maintaining the irritation, and may produce some of the secondary results. The bacillus coli, the pyogenic cocci, etc., are often present, and may extend along the urethra to the bladder and set up cystitis, though in this they may be aided by the passage of a catheter. It may be mentioned here that Wertheim cultivated the gonococcus from a case of chronic gonorrhœa of two years' standing, and by inoculation on the human subject proved it to be still virulent.

In the disease in the female, gonococci are almost invariably present in the urethra, the situation affected next in frequency being the cervix uteri. They do not appear to infect the lining epithelium of the vagina of the adult unless some other abnormal condition be present, but they do so in the gonorrhœal vulvovaginitis of young subjects. They have also been found in suppurations in connection with Bartholini's glands, and sometimes produce an inflammatory condition of the mucous membrane of the body of the uterus. They may also pass along the Fallopian tubes and produce inflammation of the mucous membrane there. From the pus in cases of pyosalpinx they have been cultivated in a considerable number of cases. According to the results of various observers they are present in one out of four or five cases of this condition, usually unassociated with other organisms. Further, in a large proportion of the cases in which the gonococcus has not been found no organisms of any kind have been obtained from the pus, and in these cases the gonococci may have been once present and have subsequently died out. Lastly, they may pass to the peritoneum and produce

peritonitis, which is usually of a local character. It is chiefly to the methods of culture supplied by Wertheim that we owe our extended knowledge of such conditions.

In *gonorrhœal conjunctivitis* the mode in which the gonococci spread through the epithelium to the subjacent connective tissue is closely analogous to what obtains in the case of the urethra. Their relation to the leucocytes in the purulent secretion is also the same. Microscopic examination of the secretion alone in acute cases often gives positive evidence, and pure cultures may be readily obtained on blood-agar. As the condition becomes more chronic the gonococci are less numerous and a greater proportion of other organisms may be present.

*Relations to Joint Affections, etc.*—The relations of the gonococcus to the sequelæ of gonorrhœa form a subject of great interest and importance, and the application of recent methods of examination shows that the organism is much more frequently present in such conditions than the earlier results indicated. The following statements may be made with regard to them. First, in a considerable number of cases of arthritis following gonorrhœa the gonococcus has been found microscopically, and pure cultures have been obtained, *e.g.* by Neisser, Lang, Bordoni-Uffreduzzi, and many others. A similar statement applies to inflammation of the sheaths of tendons following gonorrhœa. Secondly, in a large proportion of cases no organisms have been found. It is, however, possible that in a number of these the gonococci may have been present in the synovial membrane, as it has been observed that they may be much more numerous in that situation than in the fluid. Thirdly, in some cases, especially in those associated with extensive suppuration, occasionally of a pyæmic nature, various pyogenic cocci have been found to be present. In the instances in which the gonococcus has been found in the joints, the fluid present has usually been described as being of a whitish-yellow tint, somewhat turbid, and containing shreds of fibrin-like material, sometimes purulent in appearance. In one case Bordoni-Uffreduzzi cultivated the gonococcus from a joint-affection, and afterwards produced gonorrhœa in the human subject by inoculating with the cultures obtained. In another case in which pleurisy was present along with arthritis the gonococcus was cultivated from the fluid in the pleural cavity. The existence of a *gonorrhœal endocarditis* has been established by recent observations. Cases apparently of this nature occurring in the course of gonorrhœa had been previously described, but the complete bacteriological test has now been satisfied in several instances. In one case



Lenhartz produced gonorrhœa in the human subject by inoculation with the organisms obtained from the vegetations. That a true *gonorrhœal septicæmia* may also occur has also been established, cultures of the gonococcus having been obtained from the blood during life on more than one occasion (Thayer and Blumer, Thayer and Lazear, Ahmann).

**Methods of Diagnosis.**—For microscopical examination dried films of the suspected pus, etc., may be stained by any of the simple solutions of the basic aniline stains. We prefer methylene- or thionin-blue, as they do not overstain, and the films do not need to be decolorised. Staining for one minute is sufficient. It is also advisable to stain by Gram's method, and it is a good plan to put at one margin of the cover-glass a small quantity of culture of *staphylococcus aureus* if available, in order to have a standard by which to be certain that the supposed gonococci are really decolorised. Regarding the value of microscopic examination alone, we may say that the presence of a large number of micrococci in a urethral discharge having the characters, position, and staining reactions described above, is practically conclusive that the case is one of gonorrhœa. There is no other condition in which the sum total of the microscopical characters is present. We consider that it is sufficient for purposes of clinical diagnosis, and therefore of great value; in the acute stage a diagnosis can thus be made earlier than by any other method. The mistake of confusing gonorrhœa with such conditions as a urethral chancre with urethritis, will also be avoided. Even in chronic cases the typical picture is often well maintained, and microscopic examination alone may give a definite positive result. When other organisms are present, and especially when the gonococci are few in number, it is difficult, and in some cases impossible, to give a definite opinion, as a few gonococci mixed with other organisms cannot be recognised with certainty. This is often the condition in chronic gonorrhœa in the female. Microscopic examination, therefore, though often giving positive results, will sometimes be inconclusive. As regards lesions in other parts of the body microscopic examination alone is quite insufficient; it is practically impossible, for example, to distinguish by this means the gonococcus from the *diplococcus intracellularis* of meningitis. Cultures alone supply the absolute test, and when the organism is present in an apparent condition of purity, Wertheim's medium or blood-agar should be used. If other organisms are present, we are practically restricted to Wertheim's plate method.

## SOFT SORE.

The bacillus of soft sore was first described by Ducrey in 1889, who found it in the purulent discharge from the ulcerated surface; and later, in 1892, Unna described its appearance and distribution as seen in sections through the sores. The statements of these observers regarding the presence and characters of this organism have been fully confirmed by other observers.

*Microscopical Characters.*—The organism occurs in the form of minute oval rods measuring about  $1.5\ \mu$  in length, and  $.5\ \mu$  in thickness (Fig. 76). It is found mixed with other organisms in the purulent discharge from the surface, and is chiefly arranged in small groups or in short chains. When studied in sections through the ulcer it is found in the superficial part of the floor, but more deeply situated than other organisms, and may be present in a state of purity amongst the leucocytic infiltration. In this position it is usually arranged in chains which may be of considerable length, and which are often seen lying in parallel rows between the cells. The bacilli chiefly occur in the free condition, but occasionally a few may be contained within leucocytes.



FIG. 76.—Film preparation of pus from soft chancre, showing Ducrey's bacillus, chiefly arranged in pairs; stained with carbolfuchsin and slightly decolorised.  $\times 1500$ .

There is no doubt that in many cases the organism is present in the buboes in a state of purity; it has been found there by microscopic examination, and cultures have also been obtained from this source. The negative results of some observers are probably due to the organism having died off. On the whole the evidence goes to show that the ordinary bubo associated with soft sore is to be regarded as another lesion produced by Ducrey's bacillus. Sometimes the ordinary pyogenic organisms become superadded.

This bacillus takes up the basic aniline stains fairly readily,

but loses the colour very rapidly when a decolorising agent is applied. Accordingly, in film preparations when dehydration is not required, it can be readily stained by most of the ordinary combinations, though Löffler's or Kühne's methylene-blue solutions are preferable, as they do not overstain. In sections, however, great care must be taken in the process of dehydration, and the aniline-oil method (*vide* p. 93) should be used for this purpose, as alcohol decolorises the organism very readily. A little of the methylene-blue or other stain may be with advantage added to the aniline-oil used for dehydrating.

**Cultivation.**—Although for a long period of time attempts to obtain cultures were unsuccessful, success has been attained



FIG. 77.—Ducrey's bacillus from a 24-hour culture in blood-bouillon.  $\times 1500$ .<sup>1</sup>

within recent years. Benzançon, Griffon, and Le Sourd obtained pure cultures in four cases, the medium used being a mixture of rabbit's blood and agar, in the proportion of one part of the former to two of the latter. The blood is added to the agar in the melted condition at 45° C., and the tubes are then sloped. Davis confirms these results, and finds that another good medium is freshly-drawn human blood distributed in small tubes; this

method is specially suitable, as the blood inhibits the growth of various extraneous organisms. On the solid medium (blood-agar) the growth appears in the form of small round globules, which attain their complete development in forty-eight hours, having then a diameter of 1 to 2 mm.; the colonies do not become confluent. Microscopic examination of these colonies, which are dissociated with some difficulty, shows similar appearances to those observed when the organism is in the tissues (Fig. 77), but occasionally long undivided filaments are observed which Davis regards as degenerative forms. Within a comparatively short period cultures undergo marked degenerative changes, and great irregularities of form and shape are to be found. It

We are indebted to Dr. Davis for the use of Figs. 76 and 77.

would appear that a comparatively large amount of blood is necessary for the growth of this organism, and even sub-cultures on the ordinary media, including blood-serum media, give negative results. Inoculation of the ordinary laboratory animals is not attended by any result, but it has been found that some monkeys are susceptible, small ulcerations being produced by superficial inoculation, and in these the organism can be demonstrated. Tomaszewski cultivated the organism for several generations, and reproduced the disease by inoculation of the human subject. The causal relationship of this bacillus must therefore be considered as completely established, and the conditions under which it grows show it to be a strict parasite—a fact which is in conformity with the known facts as to the transmission of the disease.

### SYPHILIS.

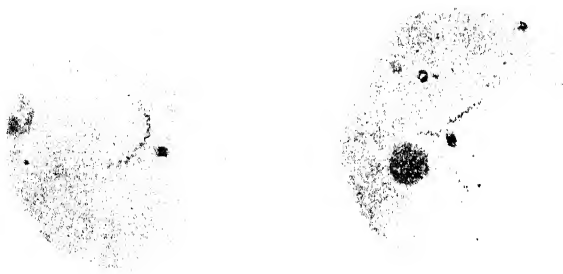
Up till quite recent times practically nothing of a definite nature was known regarding the etiology of syphilis. Most interest for a long time centred around the observations of Lustgarten, who in 1884 described a characteristic bacillus, both in the primary sore and in the lesions in internal organs. This organism occurred in the form of slender rods, straight, or slightly bent, 3 to 4  $\mu$  in length, often forming little clusters either within cells or lying free in the lymphatic spaces; it took up basic aniline dyes with some difficulty, but was much more easily decolorised by acids than the tubercle bacillus.

Lustgarten stained the tissues for twenty-four to forty-eight hours in aniline-water solution of gentian violet; and then, after washing them in alcohol, placed them for ten seconds in a 1·5 per cent solution of permanganate of potassium. They were then treated with sulphurous acid, which removes the brown precipitate formed, and decolorises the sections. They were then washed in water, dehydrated, and mounted.

Much controversy arose regarding the significance of this bacillus. Some considered it to be the tubercle bacillus, whilst others supposed that it was the smegma bacillus which had invaded the tissues. The etiological relationship of the organism to the disease was, however, not generally accepted, and in view of the recent work on syphilis, the organism cannot be regarded as having any pathological importance.

**Spirochæte pallida.**—An entirely new light has been thrown on the etiology of the disease by the work of Schaudinn and Hoffmann which appeared in 1905. Since their first publication a great amount of work has been undertaken in order to test

their conclusions, and the general result may be said to be of a confirmatory nature. These observers found in certain cases an organism to which they gave the name *spirochæte pallida*; it now also goes by the name *spironema pallidum*. As described by them, it is a minute spiral-shaped organism, showing usually from six to eight curves, though longer forms are met with; the curves are small, comparatively sharp, and regular (Figs. 78, 79). It may be said to measure 4-14  $\mu$  in length, while it is extremely thin, its thickness being only  $\cdot 25 \mu$ . In a fresh specimen, say a scraping from a chancre suspended in a little salt solution, the organism shows active movements, which are of three kinds—rotation about the long axis, gliding movements to and fro,



FIGS. 78 and 79.—Film preparations from juice of hard chancre showing spirochæte pallida,—Giemsa's stain.  $\times 1000$ . (From preparations by Dr. A. MacLennan.)

and movements of flexion of the whole body. The ends are pointed and tapering. Its detection is comparatively difficult, as the organism is feebly refractile, and more difficult to see than most other organisms; the movement of small particles in the vicinity, however, is of assistance in finding it.

In ulcerated syphilitic lesions other organisms are, of course, present, and not infrequently another spiral organism, to which the name *spirochæte refringens* has been given. This organism is usually somewhat longer, and is distinctly thicker than the spirochæte pallida. As the name implies, it is more highly refractile, and is much more easily detected than the latter organism; its curves also are opener and much less regular, and they vary in their appearance during the movements. In stained films (see p. 107) the differences between the organisms come out more distinctly, as can be gathered from the accompanying photograph (Fig. 81). The spirochæte pallida by the

Giemsa stain is coloured somewhat faintly, and of reddish tint, whilst the regular spiral twistings are preserved; the spirochæte *refringens* shows flatter, wave-like bends, and, like other organisms, is stained of a bluish tint.

By using Löffler's stain for the flagella of bacteria, Schaudinn was able to demonstrate a single delicate flagellum at each pole of the spirochæte *pallida*, while no undulating membrane could be detected; on the other hand, several other species, including the spirochæte *refringens*, showed a distinct undulating membrane. Two flagella at one pole of the spirochæte *pallida* were also seen, an appearance which Schaudinn thought might represent the commencement of longitudinal fission.

The number of publications with

regard to the distribution of the spirochæte *pallida* is already very large, and a summary of the results may be given. In the primary sore and in the related lymphatic glands, the juice of which can be conveniently obtained by means of a hypodermic syringe, the organism has been found in a very large majority of cases. It has been also obtained in the papular and roseolar eruptions, in condylomata and mucous patches—in fact, one may say generally, in all the primary and secondary lesions. It has been obtained from the spleen during life, and on a few occasions, *e.g.* by Schaudinn, also from the blood during life in secondary syphilis.

In the congenital form of the disease the organism may be present in large numbers, as was first shown by Buschke and Fischer, and by

Levaditi. In the pemphigoid bullæ, in the blood, in the internal organs, the liver, lungs, supra-renals, and even in the heart its detection may be comparatively easy, owing to the large numbers present (Fig. 80). It can readily be demonstrated



FIG. 80.—Section of spleen from a case of congenital syphilis, showing several examples of spirochæte *pallida*; Levaditi's method.  $\times 1000$ .



FIG. 81.—Spirochæte *refringens* in film preparation from a case of balanitis.  $\times 1000$ .

in sections of the organs by the method described on p. 104. In such preparations large numbers of spirochaetes, chiefly extravascular in position, can be seen, and many may occur in the interior of the more highly specialised cells, for example, liver-cells; in many cases examination has been made within so short a period after the death of the child as to practically exclude the possibility of contamination from without. It also abounds sometimes on mucous surfaces, *e.g.* of the bladder and intestine in cases of congenital syphilis. Shortly after the discovery of the organism, Metchnikoff was able to detect it in the lesions produced in monkeys by inoculation with material derived from syphilitic sores, and his observations have since been confirmed. Although various organisms may be associated with it in the lesions of the skin or mucous membranes, there is a comparative agreement amongst observers that this organism occurs alone in syphilitic lesions where the entrance of bacteria, etc., from outside is excluded. The high percentage of cases in which it is found would, in view of the difficulty in detecting it, almost point to its invariable presence, and, as a matter of fact, Schaudinn in his last series of cases, numbering over seventy, found it in all. In gummata and other tertiary lesions, however, the spirochaete has rarely, if ever, been detected, and it is probable, as Schaudinn suggests, that it has passed into some resting condition which has not yet been found. Another question of considerable importance is, as to whether this organism has been found in other conditions. Observations show that in various conditions, such as ulcerated carcinomata, balanitis, etc., spirochaetes are of comparatively common occurrence. There is no doubt whatever that the great majority of these are readily distinguishable by their appearance from the spirochaete pallida, but others resemble it closely. Hoffmann, however, who has seen many of these spirochaetes from other sources, considers that even by their microscopic appearance they are capable of being distinguished, though with considerable difficulty. It must, of course, be borne in mind that the finding of an organism in non-syphilitic lesions with exactly the same microscopical characters does not show that it is the same organism as the spirochaete pallida. It cannot be claimed that the pathological relation of this organism to the disease is absolutely demonstrated; but the facts stated are sufficient to form very strong presumptive evidence that in the spirochaete pallida we have the true cause of syphilis.

**Transmission of the Disease to Animals.**—Although various experiments had previously been from time to time

made by different observers, in some cases with reported successful result, it is to the papers of Metchnikoff and Roux (1903-5) that we owe most of our knowledge. These observers have carried on a large series of observations, and have shown that the disease can be transmitted to various species of monkeys. Of these the anthropoid apes are most susceptible, the chimpanzee being the most suitable for experimental purposes. Their results have been confirmed by Lassar, Neisser, Kraus, and others. Inoculations made by scarification resulted in the production of typical primary lesions in all of more than twenty animals used. The primary lesion is in the form of an indurated papule or of papules, in every respect resembling the human lesion. Along with this there is a marked enlargement and induration of the corresponding lymphatic glands. The primary lesion appeared on an average about thirty days after inoculation, and secondary symptoms appeared in rather more than half of the cases after a further period of rather longer duration. These were of the nature of squamous papules on the skin, mucous patches in the mouth, and sometimes palmar psoriasis. As a rule, the secondary manifestations were of a somewhat mild degree, and in no instance up to the present has any tertiary lesion been observed. By inoculation from the secondary lesions, the primary manifestations with their typical characters have been reproduced. The orang-outang has been found to be less susceptible, whilst Roux's experiments on the gorilla have been too few to admit of any conclusion. The disease may also be produced in baboons and macaques (*macacus sinicus* is one of the most susceptible), but these animals are less susceptible. In the case of many of them no result follows, and when a lesion is produced it is only of the nature of a primary papule, secondary manifestations never appearing. There is thus no doubt that the disease may be produced in apes, and, to speak generally, the severity of the affection increases according to the nearness of the relationship of the animal to the human subject.

The production of the disease, experimentally, has supplied us with some further facts regarding the nature of the virus. It has been shown repeatedly that the passage of fluid containing the virus through a Berkefeld filter deprives it completely of its infectivity. In other words, the virus does not belong to the ultra-microscopic group of organisms. The virus is also readily destroyed by heat, a temperature of 51° C. being fatal. With regard to the production of immunity, very little of a satisfactory nature has so far been established. It has been



found that the virus from a macaque monkey produces a less severe disease in the chimpanzee than the virus from the human subject, inasmuch as secondary lesions do not follow; the virus would thus appear to have undergone a certain amount of attenuation in the tissues of that monkey. Recently corneal ulcers in rabbits have been produced by Bertarelli and by Hoffmann by inoculation with syphilitic material; they appear after a long period of incubation, and the spirochæte can be demonstrated in the lesions. The effects of injecting emulsions of tertiary lesions or of serum from syphilitic patients, at the time of inoculation with the virus, appear to be practically nil; so also the employment of the virus rendered inactive by heating has apparently no influence in acting as a vaccine. There is some evidence that the serum from a patient suffering from the disease when mixed with the virus before inoculation modifies the disease to a certain extent, but further evidence on this point is necessary. As mentioned above, the spirochæte pallida has been found in the lesions in monkeys, Metchnikoff and Roux obtaining positive results in more than 75 per cent of the cases, and it is to be noted that here also the organism has been found deep in the substance of the papules, unaccompanied by any other organisms. Hoffmann failed to find any spirochætes in monkeys which had not been inoculated with syphilitic material. This observer produced a lesion on the upper eyelid of a macacus by inoculation with the blood of a man who had suffered from the disease for six months, and a papule appeared which contained spirochætes. This result is in conformity with that given by microscopic examination, and shows that the organism is sometimes present in the circulating blood in severe cases of the disease, and that the blood is accordingly infective.

Castellani has described in yaws or frambœsia the occurrence of a spirochæte closely resembling the spirochæte pallida in appearance, and to this organism he has given the name *spirochæte pertenuis*. He has found it not only in the skin lesions but also in the spleen and lymphatic glands of patients suffering from the disease. He has produced the disease in monkeys by direct inoculation and has found the spirochæte in the resulting lesions. He finds that the immunity reactions of the two organisms—spirochæte pallida and spirochæte pertenuis—are quite distinct; hence we have probably to deal with two distinct species.

## CHAPTER IX.

### TUBERCULOSIS.

THE cause of tubercle was proved by Koch in 1882 to be the organism now universally known as the tubercle bacillus. Probably no other single discovery has had a more important effect on medical science and pathology than this. It has not only shown what is the real cause of the disease, but has also supplied infallible methods for determining what are tubercular lesions and what are not, and has also given the means of studying the modes and paths of infection. A definite answer has in this way been supplied to many questions which were previously the subject of endless discussion.

**Historical.**—By the work of Armanni and of Cohnheim and Salomonsen (1870-80) it had been demonstrated that tubercle was an infective disease. The latter observers found on inoculation of the anterior chamber of the eye of rabbits with tubercular material that in many cases the results of irritation soon disappeared, but that after a period of incubation, usually about twenty-five days, small tubercular nodules appeared in the iris; afterwards the disease gradually spread, leading to a tubercular disorganisation of the globe of the eye. Later still, the lymphatic glands became involved, and finally the animal died of acute tuberculosis. The question remained as to the nature of the virus, the specific character of which was thus established, and this question was answered by the work of Koch.

The announcement of the discovery of the tubercle bacillus was made by Koch in March 1882, and a full account of his researches appeared in 1884 (*Mitth. a. d. K. Gesundheitsamte.*, Berlin). Koch's work on this subject will remain as a classical masterpiece of bacteriological research, both on account of the great difficulties which he successfully overcame and the completeness with which he demonstrated the relations of the organism to the disease. The two chief difficulties were, first, the demonstration of the bacilli in the tissues, and, secondly, the cultivation of the organism outside the body. For, with regard to the first, the tubercle bacillus cannot be demonstrated by a simple watery solution of a basic aniline dye, and it was only after prolonged staining for twenty-four hours, with a solution of methylene-blue with caustic potash added, that he was able to reveal the presence of the organism. Then, in the second place,

all attempts to cultivate it on the ordinary media failed, and he only succeeded in obtaining growth on solidified blood serum, the method of preparing which he himself devised, inoculations being made on this medium from the organs of animals artificially rendered tubercular. The fact that growth did not appear till the tenth day at the earliest, might easily have led to the hasty conclusion that no growth took place. All difficulties were, however, successfully overcome. He cultivated the organism by the above method from a great variety of sources, and by a large series of inoculation experiments on various animals, performed by different methods, he conclusively proved that bacilli from these different sources produced the same tubercular lesions and were really of the same species. His work was the means of showing conclusively that such conditions as lupus, "white swelling" of joints, scrofulous disease of glands, etc., are really tubercular in nature.

**Tuberculosis in Animals.**—Tuberculosis is not only the most widely spread of all diseases affecting the human subject, and produces a mortality greater than any other, but there is probably no other disease which affects the domestic animals so widely. We need not here describe in detail the various tubercular lesions in the human subject, but some facts regarding the disease in the lower animals may be given, as this subject is of great importance in relation to the infection of the human subject.

Amongst the domestic animals the disease is commonest in cattle (bovine tuberculosis), in which animals the lesions are very various, both in character and distribution. In most cases the lungs are affected, and contain numerous rounded nodules, many being of considerable size; these may be softened in the centre, but are usually of pretty firm consistence and may be calcified. There may be in addition caseous pneumonia, and also small tubercular granulations. Along with these changes in the lungs, the pleurae are also often affected, and show numerous nodules, some of which may be of large size, firm and pedunculated, the condition being known in Germany as *Perlsucht*, in France as *pommelière*. Lesions similar to the last may be chiefly confined to the peritoneum and pleurae. In other cases, again, the abdominal organs are principally involved. The udder becomes affected in a certain proportion of cases of tuberculosis in cows—in 3 per cent according to Bang—but primary affection of this gland is very rare. Tuberculosis is also a comparatively common disease in pigs, in which animals it in many cases affects the abdominal organs, in other cases produces a sort of caseous pneumonia, and sometimes is met with as a chronic disease of the lymphatic glands, the so-called "scrofula" of pigs. Tubercular lesions in the muscles are less rare in pigs than in most other animals. In the horse the abdominal organs are usually the primary seat of the disease, the spleen being often enormously enlarged and crowded with nodules of various shapes and sizes; sometimes, however, the primary lesions are pulmonary. In sheep and goats tuberculosis is of rare occurrence, especially in the former animals. It may occur spontaneously in dogs, cats, and in the large carnivora. It is also sometimes met with in monkeys in confinement, and leads to a very rapid and widespread affection in these animals, the nodules having a special tendency to soften and break down into a pus-like fluid.

Tuberculosis in fowls (avian tuberculosis) is a common and very infectious disease, nearly all the birds in a poultry-yard being sometimes affected.

From these statements it will be seen that the disease in animals presents great variations in character, and may differ in many respects from that met with in the human subject. The relation of the different forms of tuberculosis is discussed below.

**Tubercle Bacillus.—Microscopical Characters.**—Tubercle bacilli are minute rods which usually measure  $2\cdot5$  to  $3\cdot5\ \mu$  in length, and  $\cdot3\ \mu$  in thickness, *i.e.* in proportion to their length they are comparatively thin organisms (Figs. 82 and 83). Sometimes, however, longer forms, up to  $5\ \mu$  or more in length, are met with, both in cultures and in the tissues. They are straight or slightly curved, and are of uniform thickness, or may show slight swelling at their extremities. When stained they appear uniformly coloured, or may present small uncoloured spots along their course, with darkly-stained parts between. In such a minute organism it is extremely difficult to determine the exact nature of the unstained points. Accordingly, we find that



FIG. 82.—Tubercle bacilli, from a pure culture on glycerin agar. Stained with carbol-fuchsin.  $\times 1000$ .

some observers consider these to be spores, while others find that it is impossible to stain them by any means whatever, and consider that they are really of the nature of vacuoles. Against their being spores is also the fact that many occur in one bacillus. Others again hold that some of the condensed and highly-stained particles are spores. It is impossible to speak definitely on the question at present. We can only say that the younger bacilli stain uniformly, and that in the older forms inequality in staining is met with; this latter condition is, however, not associated with greater powers of resistance.

The bacilli in the tissues occur scattered irregularly or in little masses. They are usually single, or two are attached end to end and often form in such a case an obtuse angle. True

chains are not formed, but occasionally short filaments are met with. In cultures the bacilli form masses in which the rods are closely applied to one another and arranged in a more or less parallel manner. Tubercle bacilli are quite devoid of motility.

*Aberrant Forms.*—Though such are the characters of the organism as usually met with, other appearances are sometimes found. In old cultures, for example, very much larger elements

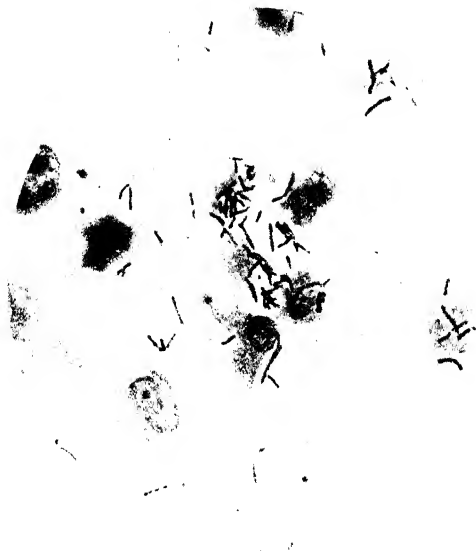


FIG. 83.--Tubercle bacilli in phthisical sputum ; they are longer than is often the case.

Film preparation, stained with carbol-fuchsin and methylene-blue.  $\times 1000$ .

may occur. These may be in the form of long filaments, sometimes swollen or clubbed at their extremities, may be irregularly beaded, and may even show the appearance of branching. Such forms have been studied by Metchnikoff, Maffucci, Klein, and others. Their significance has been variously interpreted, for while some look upon them as degenerated or involution forms, others regard them as indicating a special phase in the life history of the organism, allying it with the higher bacteria. Recent observations, however, go to establish the latter view, and this is now generally accepted by authorities. It has also

been found that under certain circumstances tubercle bacilli in the tissues produce a radiating structure closely similar to that of the actinomyces. This was found by Babes and also by Lubarsch to be the case when the bacilli were injected under the dura mater and directly into certain solid organs, such as the kidneys in the rabbit. Club-like structures may be present at the periphery; these are usually not acid-fast, but they retain the stain in the Weigert-Gram method. Similar results obtained with other acid-fast bacilli will be mentioned below, and these organisms would appear to form a group closely allied to the streptothricæ, the bacillary parasitic form being one stage of the life history of the organism. This group is often spoken of as the *mycobacteria*.

*Staining Reactions.*—The tubercle bacillus takes up the ordinary stains very slowly and faintly, and for successful staining one of the most powerful solutions ought to be employed, *e.g.* gentian-violet or fuchsin, along with aniline-oil water or solution of carbolic acid. Further, such staining solutions require to be applied for a long time, or the staining must be accelerated by heat, the solution being warmed till steam arises and the specimen allowed to remain in the hot stain for two or three minutes. One of the best and most convenient methods is the Ziehl-Neelsen method (see p. 100). The bacilli present this further peculiarity, however, that after staining has taken place they resist decolorising by solutions which readily remove the colour from the tissues and from other organisms which may be present. Such decolorising agents are sulphuric or nitric acid in 20 per cent solution. Preparations can thus be obtained in which the tubercle bacilli alone are coloured by the stain first used, and the tissues can then be coloured by a contrast stain. Within recent years certain other bacilli have been discovered which present the same staining reactions as tubercle bacilli; they are therefore called “acid-fast” (*vide infra*). The spores of many bacilli become decolorised more readily than tubercle bacilli, though some retain the colour with equal tenacity.

Bulloch and Macleod, by treating tubercle bacilli with hot alcohol and ether, extracted a wax which gave the characteristic staining reactions of the bacilli themselves. The remains of the bacilli, further, when extracted with caustic potash, yielded a body which was probably a chitin, and which was acid-fast when stained for twenty-four hours with carbol-fuchsin.

**Cultivation.**—The medium first used by Koch was inspissated blood serum (*vide p.* 39). If inoculations are made on this medium with tubercular material free from other organisms,

there appear in from ten to fourteen days minute points of growth of dull whitish colour, rather irregular, and slightly raised above the surface (it is advisable to plant on the medium an actual piece of the tubercular tissue and to fix it in a wound of the surface of the serum). Koch compared the appearance of these to that of small dry scales. In such cultures the growths usually



FIG. 84.—Cultures of tubercle bacilli on glycerin agar.

A and B. Mammalian tubercle bacilli; A is an old culture, B one of a few weeks' growth.  
C. Avian tubercle bacilli. The growth is whiter and smoother on the surface than the others.

reach only a comparatively small size and remain separate, becoming confluent only when many occur close together. In sub-cultures, however, growth is more luxuriant and may come to form a dull wrinkled film of whitish colour, which may cover the greater part of the surface of the serum and at the bottom of the tube may grow over the surface of the condensation water on to the glass (Fig. 84, A). The growth is always of a dull appearance and has a considerable degree of consistence, so that it is difficult to dissociate a portion thoroughly in a drop of water. In older cultures the growth may acquire a slightly brownish or buff colour. When the small colonies are examined under a low power of the microscope they are seen

to be extending at the periphery in the form of wavy or sinuous streaks which radiate outward and which have been compared to the flourishes of a pen. The central part shows similar markings closely interwoven. These streaks are composed of masses of the bacilli arranged in a more or less parallel manner.

On *glycerin agar*, which was first introduced by Nocard and Roux as a medium for the culture of the tubercle bacillus, growth takes place in sub-cultures at an earlier date and pro-

gresses more rapidly than on serum, but this medium is not suitable for obtaining cultures from the tissues, inoculations with tubercular material usually yielding a negative result. The growth has practically the same characters as on serum, but is more luxuriant. In *glycerin broth*, especially when the layer is not deep, tubercle bacilli grow readily in the form of little white masses which fall to the bottom and form a powdery layer. If, however, the growth be started on the surface it spreads superficially as a dull whitish, wrinkled pellicle which may reach the walls of the flask; this mode of growth is specially suitable for the production of tuberculin (*vide infra*). The culture has a peculiar fruity and not unpleasant odour. On ordinary agar and on gelatin media no growth takes place.

It was at one time believed that the tubercle bacillus would only grow on media containing animal fluids, but of late years it has been found that growth takes place also on a purely vegetable medium, as was first shown by Pawlowsky in the case of potatoes. Sander has shown that the bacillus grows readily on potato, carrot, macaroni, and on infusion of these substances, especially when glycerin is added. He also found that cultures from tubercular lesions could be obtained on glycerin potato (p. 46).

The optimum temperature for growth is  $37^{\circ}$  to  $38^{\circ}$  C. Growth ceases above  $42^{\circ}$  and usually below  $28^{\circ}$ , but on long-continued cultivation outside the body and in special circumstances, growth may take place at a lower temperature, *e.g.* Sander found that growth took place in glycerin-potato broth even at  $22^{\circ}$  to  $23^{\circ}$  C.

**Powers of Resistance.**—Tubercle bacilli have considerable powers of resistance to external influences, and can retain their vitality for a long time outside the body in various conditions; in fact, in this respect they may be said to occupy an intermediate position between spores and spore-free bacilli. Dried phthisical sputum has been found to contain still virulent bacilli (or their spores) after two months, and similar results are obtained when the bacilli are kept in distilled water for several weeks. So also they resist for a long time the action of putrefaction, which is rapidly fatal to many pathogenic organisms. Sputum has been found to contain living tubercle bacilli even after being allowed to putrefy for several weeks (Fraenkel, Baumgarten), and the bacilli have been found to be alive in tubercular organs which have been buried in the ground for a similar period. They are not killed by being exposed to the action of the gastric juice for six hours, or to a temperature of  $-3^{\circ}$  C. for three hours, even when this is repeated several times. It has been found that



when completely dried they can resist a temperature of  $100^{\circ}$  C. for an hour, but, on the other hand, exposure in the moist condition to  $70^{\circ}$  C. for the same time is usually fatal. It may be stated that raising the temperature to  $100^{\circ}$  C. kills the bacilli in fluids and in tissues, but in the case of large masses of tissue care must be taken that this temperature is reached throughout. They are killed in less than a minute by exposure to 5 per cent carbolic acid, and both Koch and Straus found that they are rapidly killed by being exposed to the action of direct sunlight.

**Action on the Tissues.**—The *local lesion* produced by the tubercle bacillus is the well-known tubercle nodule, the structure of which varies in different situations and according to the intensity of the action of the bacilli. After the bacilli gain entrance to a connective tissue such as that of the iris, their first action appears to be on the connective-tissue cells, which become somewhat swollen and undergo mitotic division, the resulting cells being distinguishable by their large size and pale nuclei—the so-called epithelioid cells. These proliferative changes may be well seen on the fifth day after inoculation or even earlier. A small focus of proliferated cells is thus formed in the neighbourhood of the bacilli and about the same time numbers of leucocytes—chiefly lymphocytes—begin to appear at the periphery and gradually become more numerous. Soon, however, the action of the bacilli as cell-poisons comes into prominence. The epithelioid cells become swollen and somewhat hyaline, their outlines become indistinct, whilst their nucleus stains faintly, and ultimately loses the power of staining. The cells in the centre, thus altered, gradually become fused into a homogeneous substance and this afterwards becomes somewhat granular in appearance. If the central necrosis does not take place quickly, then giant-cell formation may occur in the centre of the follicle, this constituting one of the characteristic features of the tubercular lesion; or after the occurrence of caseation giant-cells may be formed in the cellular tissue around. The centre of a giant-cell often shows signs of degeneration, such as hyaline change and vacuolation, or it may be more granular than the rest of the cell.

Though there has been a considerable amount of discussion as to the mode of origin of the giant-cells, we think there can be little doubt that in most cases they result from enlargement of single epithelioid cells, the nucleus of which undergoes proliferation without the protoplasm dividing. These epithelioid cells may sometimes be the lining cells of capillaries. Some consider that the giant-cells result from a fusion of the epithelioid

cells; but, though there are occasionally appearances which indicate such a mode of formation, it cannot be regarded as of common occurrence. In some cases of acute tuberculosis, when the bacilli become lodged in a capillary the endothelial cells of its wall may proliferate, and thus a ring of nuclei may be seen round a small central thrombus. Such an occurrence gives rise to an appearance closely resembling a typical giant-cell. According to the view here stated, both the epithelioid and the giant-cells are of connective tissue origin; and we can see no sufficient evidence for the view held by some observers, chiefly of the French school, that they are formed from leucocytes which have emigrated from the capillaries.

There can be no doubt that the cell necrosis and subsequent caseation depend upon the products of the bacilli, and are not due to the fact that the tubercle nodule is non-vascular. This non-vascularity itself is to be explained by the circumstance that young capillaries cannot grow into a part where tubercle bacilli are active, and that the already existing capillaries become thrombosed, owing to the action of the bacillary products on their walls, and ultimately disappear. At the periphery of tubercular lesions there may be considerable vascularity and new formation of capillaries.

The *general symptoms of tuberculosis*—pyrexia, perspiration, wasting, etc., are to be ascribed to the absorption and distribution throughout the system of the toxic products of the bacilli; in the case of phthisical cavities and like conditions where other bacteria are present, the toxins of the latter also play an important part. The occurrence of waxy change in the organs is believed by some to be chiefly due to the products of other, especially pyogenic, organisms, secondarily present in the tubercular lesions. This matter, however, requires further elucidation.

*Presence and Distribution of the Bacilli.*—A few facts may be stated regarding the presence of bacilli, and the numbers in which they are likely to be found in tubercular lesions. On the one hand, they may be very few in number and difficult to find, and on the other hand, they may be present in very large numbers, sometimes forming masses which are easily visible under the low power of the microscope.

They are usually very few in number in chronic lesions, whether these are tubercle nodules with much connective tissue formation or old caseous collections. In caseous material one can sometimes see a few bacilli faintly stained, along with very minute unequally stained granular points, some of which may possibly be spores of the bacilli. Whether they are spores or

not, the important fact has been established that tubercular material in which no bacilli can be found microscopically, may be proved, on experimental inoculation into animals, to be still virulent. In such cases the bacilli may be present in numbers so small as to escape observation, or it may be that their spores only are present. In subacute lesions, with well-formed tubercle follicles and little caseation, the bacilli are generally scanty.

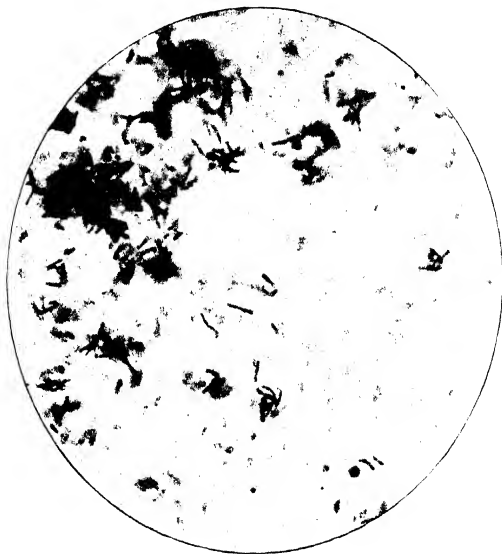


FIG. 85.—Tubercle bacilli in section of human lung in acute phthisis. The bacilli are seen lying singly, and also in large masses to left of field. The pale background is formed by caseous material.

Stained with carbol-fuchsin and Bismarck-brown.  $\times 1000$ .

They are most numerous in acute lesions, especially where caseation is rapidly spreading, for example, in such conditions as caseous catarrhal pneumonia (Fig. 85), acute tuberculosis of the spleen in children, which is often attended with a good deal of rapid caseous change, etc. In acute miliary tuberculosis a few bacilli can generally be found in the centre of the follicles; but here they are often much more scanty than one would expect. The tubercle bacillus is one which not only has comparatively slow growth, but retains its form and staining power for a much

longer period than most organisms. As a rule the bacilli are extra-cellular in position. Occasionally they occur within the giant-cells, in which they may be arranged in a somewhat radiate manner at the periphery, occasionally also in epithelioid cells and in leucocytes.

The above statements, however, apply only to tuberculosis in the human subject, and even in this case there are exceptions.



FIG. 86.—Tubercle bacilli in giant-cells, showing the radiate arrangement at the periphery of the cells. Section of tubercular udder of cow. Stained with carbol-fuchsin and Bismarck-brown.  $\times 1000$ .

In the ox, on the other hand, the presence of tubercle bacilli within giant-cells is a very common occurrence; and it is also common to find them in considerable numbers scattered irregularly throughout the cellular connective tissue of the lesions, even when there is little or no caseation present (Fig. 86).

In tuberculosis in the horse and in avian tuberculosis the numbers of bacilli may be enormous, even in lesions which are not specially acute; and considerable variation both in their number and in their site is met with in tuberculosis of other animals.

In discharges from tubercular lesions which are breaking down, tubercle bacilli are usually to be found. In the sputum of phthisical patients their presence can be demonstrated almost invariably at some period, and sometimes their numbers are very large (for method of staining see p. 101). Several examinations may, however, require to be made; this should always be done before any conclusion as to the non-tubercular nature of a case is

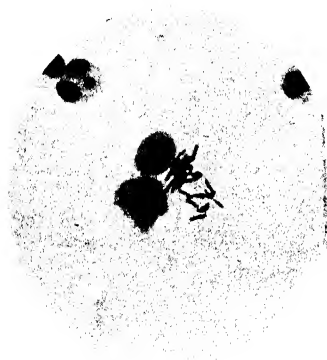


FIG. 87.—Tubercle bacilli in urine; showing one of the characteristic clumps, in which they often occur. Stained with carbol-fuchsin and methylene-blue.  $\times 1000$ .

come to. In cases of genito-urinary tuberculosis they are usually present in the urine; but as they are much diluted it is difficult to find them unless a deposit is obtained by means of the centrifuge. This deposit is examined in the same way as the sputum. The bacilli often occur in little clumps, as shown in Fig. 87. In tubercular ulceration of the intestine their presence in the faeces may be demonstrated, as

was first shown by Koch; but in this case their discovery is usually of little importance, as the intestinal lesions, as a rule, occur only in advanced stages when diagnosis is no longer a matter of doubt.

**Experimental Inoculation.**—Tuberculosis can be artificially produced in animals by infection in a great many different ways—by injection of the bacilli into the subcutaneous tissue, into the peritoneum, into the anterior chamber of the eye, into the veins; by feeding the animals with the bacilli; and, lastly, by making them inhale the bacilli suspended in the air.

The exact result, of course, varies in different animals and according to the method of inoculation, but we may state generally that when introduced into the tissues of a susceptible animal, the bacilli produce locally the lesions above described, terminating in caseation; that there occurs a tubercular affection of the neighbouring lymphatic glands, and that lastly there may be a rapid extension of the bacilli to other organs by the blood stream and the production of general tuberculosis. Of

the animals generally used for the purpose, the guinea-pig is most susceptible.

When a guinea-pig is inoculated subcutaneously with tubercle bacilli from a culture, or with material containing them, such as phthisical sputum, a local swelling gradually forms which is usually well marked about the tenth day. This swelling becomes softened and caseous, and may break down, leading to the formation of an irregularly ulcerated area with caseous lining. The lymphatic glands in relation to the parts can generally be found to be enlarged and of somewhat firm consistence, about the end of the second or third week. Later, in them also caseous change occurs, and a similar condition may spread to other groups of glands in turn, passing also to those on the other side of the body. During the occurrence of these changes, the animal loses weight, gradually becomes cachectic, and ultimately dies, sometimes within six weeks, sometimes not for two or three months. *Post mortem*, in addition to the local and glandular changes, an acute tuberculosis is usually present, the spleen being specially affected. This organ is swollen, and is studded throughout by numerous tubercle nodules, which may be minute and grey, or larger and of a yellowish tint. If death has been long delayed, calcification may have occurred in some of the nodules. Tubercle nodules, though rather less numerous, are also present in the liver and in the lungs, the nodules in the latter organs being usually of smaller size though occasionally in large numbers. The extent of the general infection varies; sometimes the chronic glandular changes constitute the outstanding feature.

*Intraperitoneal* injection of pure cultures produces a local lesion in the form of an extensive tubercular infiltration and thickening of the omentum, sometimes attended with acute tubercles all over the peritoneum. There is a caseous enlargement of the retroperitoneal and other lymphatic glands, and later there may be a general tuberculosis. *Intravenous* injection produces a typical acute tuberculosis, the nodules being usually more numerous and of smaller size, while death follows more rapidly, the larger the numbers of bacilli injected. Guinea-pigs, when fed with tubercle bacilli, or with sputum or portions of tissue containing them, readily contract an intestinal form of tuberculosis, lesions being present in the lymphoid tissue of the intestines, in the mesenteric glands, and later in the internal organs.

Rabbits are less susceptible than guinea-pigs, and in them the effects of subcutaneous inoculation are very variable; sometimes the lesions remain local, sometimes a general tuberculosis is set up. Otherwise the reactions are much of the same nature. Dogs are much more highly resistant, but tuberculosis can be produced in them by intraperitoneal injection of pure cultures (Koch), or by intravenous injection (Maffucci). In the latter case there results an extensive eruption of minute miliary

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tubercles. Tuberculosis can also be easily produced in susceptible animals by making them inhale the bacilli.

**Varieties of Tuberculosis.** 1. *Human and Bovine Tuberculosis.*—Up till recent years it was generally accepted that all mammalian tuberculosis was due to the same organism, and in particular that tuberculosis could be transmitted from the ox to the human subject. The matter became one of special interest owing to Koch's address at the Tuberculosis Congress in 1901, in which he stated his conclusion that human and bovine tuberculosis are practically distinct, and that if a susceptibility of the human subject to the latter really exists, infection is of very rare occurrence,—so rare that it is not necessary to take any measures against it. Previously to this, Theobald Smith had pointed out differences between mammalian and bovine tubercle bacilli, the most striking being that the latter possess a much higher virulence to the guinea-pig, rabbit, and other animals, and in particular that human tubercle bacilli, on inoculation into oxen, produce either no disease or only local lesions without any dissemination. Koch's conclusions were based chiefly on the result of his inoculations of the bovine species with human tubercle bacilli, the result being confirmatory of Smith's, and, secondly, on the supposition that infection of the human subject through the intestine is of very rare occurrence.

Since the time of Koch's communication an enormous amount of work has been done on this subject, and Commissions of inquiry have been appointed in various countries. We may summarise the chief facts which have been established. Practically all observers are agreed that there are two chief types of tubercle bacilli which differ both in their cultural characters and in their virulence—a bovine type and a human type. The bacilli of the bovine type when cultivated are shorter and thicker and more regular in size; whilst their growth on various culture media is scantier than that of the human type. From the latter character the British Royal Commission have applied the term *dysgonic* to the bovine and *eugonic* to the human type. As already stated there is also a great difference in virulence towards the lower animals, the bacillus from the ox having a much higher virulence. This organism when injected in suitable quantities into the ox produces a local tubercular lesion, which is usually followed by a generalised and fatal tuberculosis; whereas injection of human tubercle bacilli produces no more than a local lesion, which undergoes retrogression. (In certain experiments, *e.g.* those of Delépine, Hamilton and

Young, general tuberculosis has been produced by tubercle bacilli from the human subject, but these results are exceptional). Corresponding differences come out in the case of the rabbit; in fact, intravenous injection of suitable quantities in this animal is the readiest method of distinguishing the two types—an acute tuberculosis resulting with the bovine, but not with the human type. In guinea-pigs and monkeys a generalised tuberculosis may result from subcutaneous injection of bacilli of the human type, but in this case also the difference in favour of the greater virulence of the bovine type is made out. With regard to the distribution of the two types of organisms, it may be stated that so far as we know the bacillus obtained from bovine tuberculosis is always of the bovine type, and the same may be said to be true of tuberculosis in pigs; in fact this seems to be the prevalent organism in animal tuberculosis. In human tuberculosis the bacilli in a large majority of the cases are of the human type; but on the other hand, in a certain proportion bacilli of the bovine type are present, the bacilli when cultivated being indistinguishable by any means at our disposal from those obtained from bovine tuberculosis. The Royal Commission found the bovine type in 14 out of 60 cases of human tuberculosis—a somewhat higher proportion than has been obtained by most other investigators—and in all of these, with one exception, the bacilli were obtained either from caseous cervical glands, or from the lesions of primary abdominal tuberculosis, that is from cases where there was evidence of infection by alimentation. It is also to be noted that almost all the tubercular lesions from which the bovine type has been obtained have been in children. The general result accordingly is that bovine tubercle bacilli are present in a certain proportion of cases of tuberculosis in young subjects, and that these are especially cases where infection by the alimentary canal has occurred. It must thus be held as established that tuberculosis is transmissible from the ox to man, and that the milk of tubercular cows is a common vehicle of transmission.

Although most of the bacilli which have been cultivated correspond to one of the two types, as above described, it is also to be noted that intermediate varieties are met with. It has also been found that the type characters of the bacillus are not constant. Various observers have found it possible to modify bacilli of the human type by passing them through the bodies of certain animals, *e.g.* guinea-pigs, sheep, and goats, so that they acquire the characters of bovine bacilli. In view of these facts it is probable that bovine bacilli will undergo

corresponding modifications in the tissues of the human subject—what period of time is necessary for such a change we cannot say. It is thus possible that the cases from which the bovine type has been obtained do not represent the full number where infection from the ox has occurred. It is quite likely that although the bovine bacilli are more virulent to the lower animals than the human bacilli are, this does not also hold in the case of the human subject. In fact the comparative chronicity of the primary abdominal lesions in children in the first instance would point rather to a low order of virulence towards the human subject. We may also add that there are cases, notably those of Ravenel, in which accidental inoculation of the human subject with bovine tubercle has resulted in the production of tuberculosis.

2. *Avian Tuberculosis*.—In the tubercular lesions in birds there are found bacilli which correspond in their staining reactions and in their morphological characters with those in mammals, but differences are observed in cultures, and also on experimental inoculation. These differences were first described by Maffucci and by Rivolta, but special attention was drawn to the subject by a paper read by Koch at the International Medical Congress in 1890. Koch stated that he had failed to change the one variety of tubercle bacillus into the other, though he did not conclude therefrom that they were quite distinct species. The following points of difference may be noted :—

On glycerin agar and on serum, the growth of tubercle bacilli from birds is more luxuriant, has a moister appearance (Fig. 84, C), and, moreover, takes place at a higher temperature, 43·5° C., than is the case with ordinary tubercle bacilli. Experimental inoculation brings out even more distinct differences. Tubercle bacilli derived from the human subject, for example, when injected into birds, usually fail to produce tuberculosis, whilst those of avian origin very readily do so. Birds are also very susceptible to the disease when fed with portions of the organs of birds containing tubercle bacilli, but they can consume enormous quantities of phthisical sputum without becoming tubercular (Straus, Wurtz, Nocard). No doubt, on the other hand, there are cases on record in which the source of infection of a poultry-yard has apparently been the sputum of phthisical patients. Again, tubercle bacilli cultivated from birds have not the same effect on inoculation of mammals as ordinary tubercle bacilli have. When guinea-pigs are inoculated subcutaneously they usually resist infection, though occasionally a fatal result follows. In the latter case, usually no tubercles visible to the naked eye are found, but numerous bacilli may be present in internal organs, especially in the spleen, which is much swollen. Further, intravenous injection even of large quantities of avian tubercle bacilli, in the case of dogs, leads to no effect, whereas ordinary tubercle bacilli produce acute tuberculosis. [The rabbit, on the other hand, is comparatively susceptible to avian tuberculosis (Nocard).]

There is, therefore, abundant evidence that the bacilli derived from the two classes of animals show important differences, and, reasoning from analogy, we might infer that probably the human subject also would be little susceptible to infection from avian tuberculosis. The question remains, are these differences of a permanent character? The matter seems conclusively settled by the experiments of Nocard, in which mammalian tubercle bacilli have been made to acquire all the characters of those of avian origin. The method adopted was to place bacilli from human tuberculosis in small collodion sacs (*v. p.* 123) containing bouillon, and then to insert each sac in the peritoneal cavity of a fowl. The sacs were left *in situ* for periods of from four to eight months. They were then removed, cultures were made from their contents, fresh sacs were inoculated from these cultures and introduced into other fowls. In such conditions the bacilli are subjected only to the tissue juices, the wall of the sac being impervious both to bacilli and to leucocytes, etc. After one sojourn of this kind, and still more so after two, the bacilli are found to have acquired some of the characters of avian tubercle bacilli, but are still non-virulent to fowls. After the third sojourn, however, they have acquired this property, and produce in fowls the same lesion as bacilli derived from avian tuberculosis. It therefore appears that the bacilli of avian tuberculosis are not a distinct and permanent species, but a variety which has been modified by growth in the tissues of the bird. Evidently also there are degrees of this modification according to the period of time during which the bacilli have passed from bird to bird, as in some cases inoculation with tubercle bacilli of avian origin has produced ordinary tubercle nodules in guinea-pigs (Courmont and Dor). It is also interesting to note that Rabinowitch has cultivated tubercle bacilli of the mammalian type from some cases of tuberculosis in parrots kept in confinement.

3. *Tuberculosis in the Fish*.—Bataillon, Dubard, and Terre cultivated from a tubercle-like disease in a carp, a bacillus which, in staining reaction and microscopic characters, closely agrees with the tubercle bacillus. The lesion with which it was associated was an abundant growth of granulation tissue in which numerous giant-cells were present. It forms, however, luxuriant growth at the room temperature, the growth being thick and moist like that of avian tubercle bacilli (Fig. 89, c). Growth does not occur at the body temperature, though by gradual acclimatisation a small amount of growth has been obtained up to 36° C. Furthermore, the organism appears to

undergo no multiplication when injected into the tissues of mammals, and attempts to modify this characteristic have so far been unsuccessful. Weber and Taute have cultivated this organism from mud, and also from organs of healthy frogs. It is thus probably to be regarded as a saprophyte which is only occasionally associated with disease in the fish. According to the results of different experimenters it is possible to modify human tubercle bacilli by allowing them to sojourn in the tissues of cold-blooded animals, *e.g.* the frog, blind-worm, etc., so that they flourish at lower temperatures. These results have, however, been recently called in question, as it has been stated the organisms obtained were not modified tubercle bacilli but other acid-fast bacilli which may be found in the tissues of normal cold-blooded animals. This question must accordingly be considered still an open one.

All the above facts taken together indicate that tubercle bacilli may become modified in relative virulence and in conditions of growth by sojourn in the tissues of various animals. This modification appears slight, though of definite character in the case of bovine tuberculosis, more distinct in the case of avian tuberculosis, and much more marked, if not permanent, in the case of fish tuberculosis, that is, of course, in their relations to the bacilli from the human subject.

**Other Acid-fast Bacilli.**—Within recent years a number of bacilli presenting the same staining reaction as the tubercle bacilli have been discovered. Such bacilli have a comparatively wide distribution in nature, as they have been obtained from various species of grass, from butter and milk, from manure, and from the surfaces of animal bodies. Microscopically, they agree more or less closely with tubercle bacilli, though most of them are shorter and plumper; many of them show filamentous and branching forms under certain conditions of culture. Moreover, on injection, they produce granulation-tissue nodules which may closely resemble tubercles, although on the whole there is a greater tendency to softening and suppuration, and usually the lesions are localised to the site of inoculation. The most important point of distinction is the fact that their multiplication on artificial media is much more rapid, growth usually being visible within forty-eight hours and often within twenty-four hours at 37° C. Furthermore, in most instances, growth occurs at the room temperature. The general character of the cultures in this group is a somewhat irregular layer, often with wrinkled surface, dry or moist in appearance, and varying in tint from white to yellow or reddish brown. The number of such

organisms is constantly being added to, but the following may be mentioned as examples:—

*Moeller's Grass Bacilli, I. and II.*—The former was found in infusions of Timothy-grass (*Phleum pratense*). It is extremely acid-fast, morphologically resembles the tubercle bacillus, and in cultures may show club formation and branching. The lesions produced closely resemble tubercles. The colonies, visible in thirty-six hours, are scale-like and of greyish-white colour (Fig. 89, *a*). Moeller's bacillus II. was obtained from the dust of a hay-loft. The colonies at first are moist and somewhat tenacious, but afterwards run together, and are of a dull yellowish colour. The general results of inoculation resemble those of grass

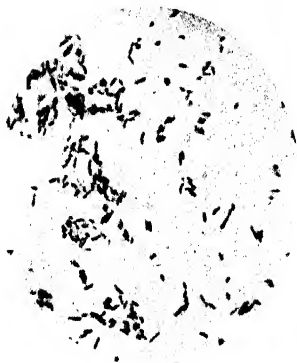


FIG. 88.—Moeller's Timothy-grass bacillus.  
From a culture on agar.  
Stained with carbol-fuchsin, and treated with  
20 per cent sulphuric acid.  $\times 1000$ .

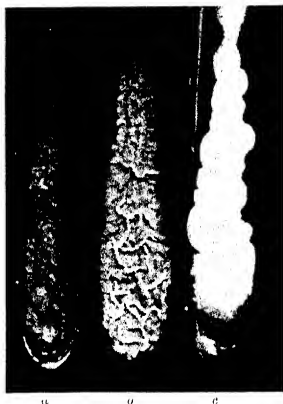


FIG. 89.—Cultures of acid-fast bacilli  
grown at room temperature.

- (a) Moeller's Timothy-grass bacillus I.
- (b) The Petri-Rabinowitch butter bacillus.
- (c) Bacillus of fish tuberculosis.

bacillus I. but are less marked. Moeller also obtained a similar organism from milk. He also discovered a third acid-fast bacillus which he obtained from manure and therefore called the "Mistbacillus" (dung bacillus). This organism has analogous characters, though presenting minor differences. It also has pathogenic effects.

Petri and Rabinowitch independently cultivated an acid-fast bacillus from butter ("butter bacillus") in which it occurs with comparative frequency. The organism resembles the tubercle bacillus, although it is on the whole shorter and thicker. Its lesions closely resemble tuberculosis, especially when injection of the organism is made into the peritoneal cavity of guinea-pigs, along with butter,—the method usually adopted in searching for tubercle bacilli in butter. This organism produces pretty rapidly a wrinkled growth (Fig. 89, *b*) not unlike that of Moeller's grass, bacillus II. Korn has also obtained other two bacilli from butter which he holds to be distinct from one another and

from Rabinowitch's bacillus. The points of distinction are of a minor character. Other more or less similar bacilli have been cultivated by Tobler, Coggi, and others.<sup>1</sup>

Another bacillus of considerable interest is Johne's bacillus or the bacillus of "chronic bovine pseudo-tuberculous enteritis," the lesions produced by it being corrugated thickenings of the mucous membrane, especially of the small intestine. The disease has now been observed in various countries, and several cases in Britain have been recorded by M'Fadyean. The bacilli occur in large numbers in the lesions, and can readily be found in scrapings from the surface. They resemble the

tubercle bacillus in appearance, but on the whole are rather shorter; they are equally acid-fast. The organism has not yet been cultivated outside the body.



FIG. 90.—Smegma bacilli. Film preparation of smegma. Ziehl-Neelsen stain.  $\times 1000$ .

**Smegma Bacillus.**—This organism is of importance, as in form and staining reaction it somewhat resembles the tubercle bacillus and may be mistaken for it. It occurs often in large numbers in the smegma preputiale and in the region of the external genitals, especially where there is an accumulation of fatty matter from the secretions. Morphologically it is a slender slightly curved organism, like the tubercle bacillus but usually distinctly shorter (Fig. 90). Like the tubercle bacillus it stains with some difficulty

and resists decolorisation with strong mineral acids. Most observers ascribe the latter fact to the fatty matter with which it is surrounded, and find that if the specimen is treated with alcohol the organism is easily decolorised. Czaplewski, however, who claims to have cultivated it on various media, finds that in culture it shows resistance to decolorisation both with alcohol and with acids, and considers, therefore, that the reaction is not due to the surrounding fatty medium. We have found that in smegma it can be readily decolorised by a minute's exposure to alcohol after the usual treatment with sulphuric acid, and thus can be readily distinguished from the tubercle bacillus. We, moreover, believe that minor points of difference in the microscopic appearances of the two organisms are quite sufficient to make the experienced observer suspicious if he should meet with the smegma bacillus in urine, and lead him to apply the decolorising test. Difficulty will only occur when a few scattered bacilli retaining the fuchsin occur.

Its cultivation, which is attended with some difficulty, was first

<sup>1</sup> For further details on this subject, *vide* Potet, *Études sur les bacilles dites acidophiles*. Paris, 1902.

effected by Czaplewski. On serum it grows in the form of yellowish-grey irregularly rounded colonies about 1 mm. in diameter, sometimes becoming confluent to form a comparatively thick layer. He found that it also grew on glycerin agar and in bouillon. It is non-pathogenic to various animals which have been tested.

Cowie has recently found that acid-fast bacilli are of common occurrence in the secretions of the external genitals, mammæ, etc., in certain of the lower animals, and that these organisms vary in appearance. He considers that the term "*smegma bacillus*" probably represents a number of allied species.

The question may be asked—do these results modify the validity of the staining reaction of tubercle bacilli as a means of diagnosis? The source of any acid-fast bacilli in question is manifestly of importance, and it may be stated that when these have been obtained from some source outside the body, or where contamination from without has been possible, their recognition as tubercle bacilli cannot be established by microscopic examination alone. In the case of material coming from the interior of the body, however,—sputum, etc.,—the condition must be looked on as different, and although an acid-fast bacillus (not tubercle) has been found by Rabinowitch in a case of pulmonary gangrene we have no sufficient data for saying that acid-fast bacilli other than the tubercle bacillus flourish within the tissues of the human body except in such rare instances as to be practically negligible. (To this statement the case of the leprosy bacillus is of course an exception.) Accordingly, up till now, the microscopic examination of sputum, etc., cannot be said to have its validity shaken, and we have the results of enormous clinical experience that such examination is practically of unvarying value. Nevertheless the facts established with regard to other acid-fast bacilli must be kept carefully in view, and great care must be exercised when only one or two bacilli are found, especially if they deviate in their morphological characters from the tubercle bacillus.

**Action of dead Tubercle Bacilli.**—The remarkable fact has been established by independent investigators that tubercle bacilli in the dead condition, when introduced into the tissues in sufficient numbers, can produce tubercle-like nodules. Prudden and Hodenpyl, by intravenous injection in rabbits of cultures sterilised by heat, produced in the lungs small nodules in which giant-cells, but no caseation, were occasionally present, and which were characterised by more growth of fibrous tissue than in ordinary tubercle. The subject was very fully investigated with confirmatory results by Straus and Gamaleia, who found that, if the number of bacilli introduced into the circulation were large, there resulted very numerous tubercle nodules with well-



formed giant-cells, and occasionally traces of caseation. The bacilli can be well recognised in the nodules by the ordinary staining method. In these experiments the bacilli were killed by exposure to a temperature of  $115^{\circ}$  C. for ten minutes before being injected. Similar nodules can be produced by intraperitoneal injection. Subcutaneous injection, on the other hand, produces a local abscess, but in this case no secondary tubercles are found in the internal organs. Further, in many of the animals inoculated by the various methods a condition of marasmus sets in and gradually leads to a fatal result, there being great emaciation before death. These experiments, which have been confirmed by other observers, show that even after the bacilli are dead they preserve their staining reactions in the tissues for a long time, and also that there are apparently contained in the bodies of the dead bacilli certain substances which act locally, producing proliferative and, to a less extent, degenerative changes, and which also markedly affect the general nutrition. S. Stockman has found that an animal inoculated with large numbers of dead tubercle bacilli afterwards gives the tuberculin reaction.

**Practical Conclusions.**—From the facts above stated with regard to the conditions of growth of the tubercle bacilli, their powers of resistance, and the paths by which they can enter the body and produce disease (as shown by experiment), the manner by which tuberculosis is naturally transmitted can be readily understood. Though the experiments of Sander show that tubercle bacilli can multiply on vegetable media to a certain extent at warm summer temperature, it is doubtful whether all the conditions necessary for growth are provided to any extent in nature. At any rate, the great multiplying ground of tubercle bacilli is the animal body, and tubercular tissues and secretions containing the bacilli are the chief, if not the only, means by which the disease is spread. The tubercle bacilli leave the body in large numbers in the sputum of phthisical patients, and when the sputum becomes dried and pulverised they are set free in the air. Their powers of resistance in this condition have already been stated. As examples of the extent to which this takes place, it may be said that their presence in the air of rooms containing phthisical patients has been repeatedly demonstrated. Williams placed glass plates covered with glycerine in the ventilating shaft of the Brompton Hospital, and after five days found, by microscopic examination, tubercle bacilli on the surface, whilst Klein found that guinea-pigs kept in the ventilating shaft became tubercular. Cornet produced tuberculosis in rabbits by

~~inoculating them with dust collected from the walls of a consumptive ward.~~ Tubercle bacilli are also discharged in considerable quantities in the urine in tubercular disease of the urinary tract, and also by the bowel when there is tubercular ulceration; but, so far as the human subject is concerned, the great means of disseminating the bacilli in the outer world is dried phthisical sputum, and the source of danger from this means can scarcely be over-estimated. Every phthisical patient ought to be looked upon as a fruitful source of infection to those around, and should only expectorate on to pieces of rag which are afterwards to be burnt, or into special receptacles which are to be then sterilised either by boiling or by the addition of a 5 per cent solution of carbolic acid.

Another great source of infection is in all probability the milk of cows affected with tuberculosis of the udder. In such cases the presence of tubercle bacilli in the milk can usually be readily detected by centrifugalising it, and then examining the deposit microscopically, or by inoculating an animal with it. As pointed out by Woodhead and others, the milk from cows thus affected is probably the great source of tabes mesenterica, which is so common in young subjects. In these cases there may be tubercular ulceration of the intestine, or it may be absent. Woodhead found that out of 127 cases of tuberculosis in children, the mesenteric glands showed tubercular affection in 100, and that there was ulceration of the intestine in 43. It is especially in children that this mode of infection occurs, as in the adult ulceration of the intestine is rare as a primary infection, though it is common in phthisical patients as the result of infection by the bacilli in the sputum which has been swallowed. There is less risk of infection by means of the flesh of tubercular animals, for, in the first place, tuberculosis of the muscles of oxen being very rare, there is little chance of the bacilli being present in the flesh unless the surface has been smeared with the juice of the tubercular organs, as in the process of cutting up the parts; and in the second place, even when present they will be destroyed if the meat is thoroughly cooked.

We may state, therefore, that the two great modes of infection are by inhalation, and by ingestion, of tubercle bacilli. By the former method the tubercle bacilli will in most cases be derived from the human subject; in the latter, probably from tubercular cows, though inhaled tubercle bacilli may also be swallowed and contamination of food by tubercular material from the human subject may occur. Alike when inhaled and when ingested, tubercle bacilli may lodge about the pharynx and thus come to

infect the pharyngeal lymphoid tissue, tonsils, etc., tubercular lesions of these parts being much more frequent than was formerly supposed. Thence the cervical lymphatic glands may become infected, and afterwards other groups of glands, bones, or joints, and internal organs.

**The Toxins of the Tubercle Bacillus.**—Two outstanding features of the action of the tubercle bacillus are the occurrence of necrosis in the cells of tubercle nodules and the production of general disturbances of metabolism accompanied by fever. It is natural to refer these phenomena to the effects of toxins formed by the organism. The study of such toxins centres round the substance known as *tuberculin* which Koch brought forward in 1890-1 as a curative agent for tubercular affections.

*Koch's Tuberculin.*—Koch stated that if in a guinea-pig suffering from the effects of a subcutaneous inoculation with tubercle bacilli, a second subcutaneous inoculation of tubercle bacilli was practised in another part of the body, superficial ulceration occurred in the primary tubercular nodule, the wound healed, and the animal did not succumb to tuberculosis. This reaction was further studied by means of tuberculin, which consisted of a concentrated glycerin bouillon culture of tubercle in which the bacilli had been killed by heat. Its essential components probably were the dead and often macerated bacilli and the substances indestructible by boiling which existed in these bacilli, or which were formed during their growth. The injection of .25 c.c. of tuberculin into a healthy man causes, in from three to four hours, malaise, tendency to cough, laboured breathing, and moderate pyrexia; all of which pass off in twenty-four hours. The injection (the site of the injection being quite unimportant), however, of .01 c.c. into a tubercular person gives rise to similar symptoms, but in a much more aggravated form, and in addition there occurs around any tubercular focus great inflammatory reaction, resulting in necrosis and a casting off of the tubercular mass, when this is possible, as for instance in the case of lupus. The bacilli are, it was shown, not killed in the process.

Koch's theory of the action of the substance was that the tubercle bacillus ordinarily secretes a body having a necrotic action on the tissues. When this is injected into a tubercular patient, the proportion present round a tubercular focus is suddenly increased, inflammatory reaction takes place around, and necrosis of the spreading margin occurs very rapidly, the material containing the living or dead bacilli being thrown off en masse instead of being disintegrated piecemeal. It appears, however, that this explanation may not be the true one; for, on the one hand, other substances besides products of the tubercle bacillus may

give rise to similar effects in tubercular animals, and, on the other, a similar reaction can take place in other diseases where there is locally in the body a deposit of new tissue. Matthes has, for instance, found that albumoses and peptones isolated from the ordinary peptic digestion of various albumins give the same reaction in tubercular guinea-pigs. The injection of milk, lactic acid, ricin, all give a similar result. Before the discovery of tuberculin, Gamaleia had found that tubercular animals were very susceptible to the toxins of the vibrio Metchnikovi; and later Metchnikoff found that a similar susceptibility existed towards the toxins of the bacillus of fowl cholera. How complicated the tuberculin reaction is is shown by the fact that a similar reaction has taken place when tuberculin has been injected into persons suffering from diseases other than tubercle, *e.g.* cancer, sarcoma, syphilis.

The hopes which the introduction of tuberculin raised, that a curative agent against tuberculosis had been discovered, were soon found not to be justified. It was very difficult to see how the necrosed material which was produced and which contained the still living bacilli, could be got rid of either naturally, as would be necessary in the case of a small tubercular deposit in a lung or a lymphatic gland, or artificially, as in a complicated joint-cavity where surgical interference could be undertaken. Not only so, but the ulceration which might be the sequel of the necrosis appeared to open a path for fresh infection. Soon facts were reported which justified these criticisms. Cases where rapid acute tubercular conditions ensued on the use of tuberculin were reported, and in a few months the treatment was practically abandoned.

*The Use of Tuberculin in the Diagnosis of Tuberculosis in Cattle.*—This is now the chief use to which tuberculin is put. In cattle, tuberculosis may be present without giving rise to apparent symptoms. It is thus important from the point of view of human infection that an early diagnosis should be made. The method is applied as follows:—The animals are kept twenty-four hours in their stalls, and the temperature is taken every three hours, from four hours before the injection till twenty-four after. The average temperature in cattle is 102.2° F.; 30 to 40 centigrammes of tuberculin are injected, and if the animal be tubercular the temperature rises 2' or 3° F. in eight to twelve hours and continues elevated for ten to twelve hours. Bang, who has worked most at the subject, lays down the principle that the more nearly the temperature approaches 104° F. the more reason for suspicion is there. He gives a record of 280 cases where the value of the method was tested by subsequent *post-mortem* examination. He found that with proper precautions the error was only 3.3 per cent. The method has been largely practised in all parts of the world, and is of great value.

While it is undoubted that tuberculin contains toxic products formed by the bacilli, we know nothing of the nature of the toxins present. From the fact that filtered cultures cause little

toxic effect, and that trituration of the bacilli increases the poisonous content of a culture, it is inferred that we have to deal with endotoxins, but beyond this statement we cannot go. Hitherto no success has attended attempts to gain a closer knowledge of the nature of such substances. It has been stated that albumoses of a special kind are present in tuberculin, but nothing definite has emerged from the investigation of these bodies.

**Active Immunisation against the Tubercle Bacillus.—**

*Koch's Tuberculin-R.* Our knowledge here centres round the substance introduced by Koch in 1897 under the name of "Tuberculin-R," or the new tuberculin. Koch's new researches consisted (1) of an attempt to immunise animals against the tubercle bacillus by employing its intracellular toxins; (2) of trying to utilise such an immunisation to aid the tissues of an animal already attacked with tubercle the better to combat the effects of the bacilli. The method of obtaining the intracellular toxins was as follows. Bacilli from young virulent cultures were dried *in vacuo*, and disintegrated in an agate mill, treated with distilled water and centrifugalised. The clear fluid was decanted, and is called by Koch "Tuberculin-O." The remaining deposit was again dried, ground, treated with water and centrifugalised, the clear fluid being again decanted, and this process was repeated with successive residues till no residue remained. These fluids put together constitute the "Tuberculin-R."

From the fact that tuberculin-O gave no cloudiness when glycerin was added, Koch concluded that it contained the substances present in the glycerin-bouillon extracts originally used by him, and he held this was borne out by the readiness with which a tuberculin reaction could be caused by it. Similarly, as tuberculin-R gave a cloudiness with glycerin and did not readily originate a reaction, he considered that it contained different products of the bacillus. When injected into animals in repeated and increasing doses,  $\frac{1}{500}$  mgrm. being the initial dose, tuberculin-R is said to produce immunity against the original extract, against tuberculin-O, and against living and virulent tubercle bacilli. Another preparation has also been introduced known as "Koch's new tuberculin" (*Bazillenemulsion*). This is an emulsion of ground tubercle bacilli in water containing 50 per cent of glycerin; it thus really contains both tuberculin-O and tuberculin-R. Both, especially tuberculin-R, have been used for the treatment of tuberculosis in man, especially for early localised lesions. In the case of both substances commencing with from  $\frac{1}{400}$  to  $\frac{1}{500}$  mgrm, gradually increasing doses were given every

second day, and the rule originally laid down for the regulation of the dosage was that no amount should be given which raised the temperature more than  $0.5^{\circ}$  F. Very various opinions have been expressed as to the efficacy of such treatment. There is little doubt that in certain cases of local conditions, such as lupus, tubercular joints, glands and genito-urinary tuberculosis, improvement has followed its application; but where febrile conditions indicate that general disturbances are in existence, there is little or no justification for its being applied, and even in many local conditions the absence of benefit is so marked that by many physicians the method has been abandoned.

*Active Immunisation associated with Opsonic Observations.*—

Within recent years attention has been directed to the possibility of controlling the use of tuberculin-R by observations of its effect on the opsonic qualities of the serum. Wright, early in his work, showed that tubercle bacilli when sensitised by an appropriate serum, were readily phagocyted by the polymorphonucleate leucocytes, and the relative sensitising capacities of serum from tubercular and non-tubercular cases has been widely studied. According to Wright, in strictly localised tuberculosis the opsonic index is persistently low, varying from  $0.1$  to  $0.9$ , while in tubercle with general disturbances it fluctuates greatly from day to day, being sometimes below, sometimes above unity. To take the former and simpler case, he holds that if the treatment with injections of tuberculin-R be controlled by noting the effect produced on the opsonic index, great improvement in the patient's condition may result. Wright's interpretation of what occurs is bound up with his views on the nature of the effects produced. These views are briefly as follows. For reasons unknown the opsonic qualities of the body fluids may become abnormally low, and the tubercle bacilli, if they gain admission to the body, can multiply locally. This multiplication is associated with a still further local diminution of the opsonins. By the introduction of such a substance as Koch's tuberculin-R the bodily mechanism, whatever it is, which produces the opsonins is stimulated, and a rise in the general opsonic index occurs. Naturally this is accompanied by a passing to the site of infection of fluids more rich in opsonins than previously, the activity of the phagocytes comes into play and the tubercle bacilli are destroyed. But any such vaccination process must be controlled by constant observations of the opsonic index, and it is only by this means that not only good results can be obtained, but that the production of harmful effects can be prevented. The reason of this is that in a great many cases the injection of a bacterial

vaccine is followed by a decrease in the opsonic qualities of the serum,—the occurrence of a negative phase. During such a period of depression there is probably an increased susceptibility to the action of the bacilli. Now, in order to get permanent benefit from the vaccination process, repeated injections of the tuberculin must be practised, and if an injection be given during a negative phase, actual harm may be done. The course of a successful vaccination is that, after the passing off of the negative phase, the opsonic index should rise to above its original level,—the occurrence of a positive phase. It is when this positive phase is fully developed that a fresh inoculation can be practised with success. The new negative phase which will now occur may not cause a drop to below the level of the original state of the serum, and the hope is that its succeeding positive phase will carry the opsonic index still higher and ensure a still greater resistance to the bacterium. The importance of the observations of the opsonic index lies in this that in antibacterial vaccinations the degree of active immunisation which can be attained is always much less than is the case with immunisation against such a substance as the diphtheria toxin, although in the latter there also occur negative and positive phases of a precisely similar character. If an injection be practised during a negative phase, then a still further drop in the opsonic content of the serum will occur and a fresh growth of the invading bacilli is likely. There are very great variations in the capacities shown by tubercular patients to react to a vaccination process. In certain cases good positive phases are readily and quickly produced, while in others after an inoculation the negative phase is long continued and may even show no tendency to pass into a positive phase. The irregularities in the opsonic index in cases where there is a general disturbance of metabolism Wright explains by supposing that they result from very irregular auto-infections of the patient's body by tubercular products from the local lesions,—positive and negative phases being produced without the purposive quality which ought to characterise a successful therapeutic vaccination. Such auto-infections may come about in various ways, and Wright is of opinion that exercise, for instance, may disseminate both tubercular products and tubercle bacilli,—he having noticed in tubercle patients a fall in the opsonic index after muscular exertion.

With regard to the details of the immunisation, Wright's chief point is that the repeated, uncontrolled injections of tuberculin such as were originally given may very likely have a harmful result, and that when an injection is practised it is not necessary

for constitutional effects to occur in order that a beneficial result may follow. Hence much smaller doses of tuberculin than hitherto are given by him. For ordinary cases with low opsonic index and no evidence of constitutional disturbance, an amount of tuberculin corresponding to from one-thousandth to a six-hundredth of a millegramme of tubercle powder is a sufficient dose, and if any dose seems to produce a pronounced negative phase then a smaller dose ought to be tried at the next inoculation. For cases clinically tubercular where the index is about normal, then smaller doses, say, the equivalent of a two-thousandth of a millegramme or less ought to be used,—the effect on the index being carefully watched. In any case, the dose which is found to give the highest positive phase is the optimum dose and one which need not necessarily be increased. Cases where there is constitutional disturbance should be as a rule left untreated.

The general position of Wright and his school is, that it is only by the observation of the opsonic index that the application of the tuberculin treatment can be effectually controlled,—deductions based on clinical data, such as absence of interference with pulse rate, temperature, etc., or increase of body weight, after an inoculation being unreliable, and further evidence of the unreliability of such tests is brought forward in the fact that, in cases of apparent benefit from sanatorium treatment, the opsonic index may still be very low. With regard to the results obtained, many cases have been brought forward by Wright and others where benefit has followed the putting into practice of the principles enunciated, and there is little doubt that the work done has given a fresh start to the active immunisation method in the treatment of tuberculosis. An outstanding event of Wright's work in this field has been his insistence on the good effects produced by extremely small doses of tuberculin (down to the four-thousandth of a millegramme) given at fairly long intervals (say 10 days or more). With regard to the efficacy of the opsonic method as affording an index to the progress of a case it must be recognised that the method is still on its trial, and it is doubtful if even in the work of the most careful observers the limits of the experimental error of the opsonic method have been sufficiently defined.

The whole question of immunisation against the tubercle bacillus presents many difficulties, and it is the merit of Wright's work that it has shed fresh light on some of these. One great difficulty arises from the great chronicity of the results of the infection in the majority of human cases. It is probably



true not only of man but of many species of animals used in experimental inquiries, that many individuals are on the borderline between immunity and susceptibility. From the wide spread of the bacilli in centres of human population, it is certain that the opportunity for infection arises in a very large proportion of the race; in many cases no results follow infection, and in many others small lesions occur which do not develop further; this has actually been shown by morbid anatomists to be the case. The disease being thus so often characterised by transient local effects without constitutional disturbance, the course of an immunisation may be expected to be rather different from that obtaining in an ordinary acute affection, though the underlying processes may be of the same nature. It is difficult, for instance, on account of the slowness of tubercular processes, to define recovery from an attack of the disease, or to speak of an animal recovering from the effect of an inoculation during an immunisation. It follows that little is known regarding an attenuation of the tubercle bacillus analogous to what is an important feature in immunisations against other organisms. It has been thought by some that the tubercle bacilli from so-called scrofulous glands are less virulent than those, say, from phthisis, but apparently here sufficient attention has not been paid to the difference of the numbers of bacilli injected in each case, and this appears to be a very important point. Experiments have also been brought forward which appear to show that the injection of bacilli from avian tuberculosis could protect the dog against bacilli derived from man. But these are not yet conclusive.

**Agglutinative Phenomena.**—The serum of tubercular patients has been found to exert an agglutinating action on the tubercle bacillus. A convenient method is to add different amounts of serum, commencing with, say, .1 c.c., to quantities of a dilution of the new tuberculin (Bazillenemulsion) equivalent to 1 part of the bacterial bodies to 10,000 of diluent, and leave the mixture for 24 hours before observing. As with other agglutinative observations, it is difficult to correlate the degree of agglutinating power of the serum with the degree of immunisation possessed by the individual from which it was derived.

**Antitubercular Sera.**—Several attempts have been made to treat tuberculosis with the serum of animals immunised by the tubercle bacillus or its products. The most successful is perhaps that of Maragliano. This author distinguishes between the toxic materials contained in the bodies of the bacilli (which withstand, unchanged, a temperature of 100° C.) and those secreted into the culture fluid (which are destroyed by heat).

The substance used by him for immunising his animals consists of three parts of the former and one of the latter. The animals employed are the dog, the ass, the horse. The serum obtained from these is capable of protecting healthy animals against an otherwise fatal dose of tuberculin, but very little importance can be attached to this result. Maragliano does not appear to have studied the effects of this serum on tubercular animals, but it has been tried in a great number of cases of human tuberculosis, 2 c.c. being injected subcutaneously every two days. Improvement is said to have taken place in a certain proportion, especially of mild non-febrile cases.

An antitubercular serum has also been introduced by Marmorek. This observer considers that the tubercle bacillus cannot produce in ordinary media the toxins which it originates when exposed to the antagonism of the bodily cells. He tries to make good this defect by first growing it in a serum antagonistic to some of the phagocytic cells of the body; for this a leucotoxic serum is used. When the bacillus has grown in this presumably favourable soil it is transferred to a medium containing a substance which may be unfavourable; and for this there is employed a medium containing liver extract, the liver being an organ in which in man tubercular lesions are comparatively rare. The bacilli being thus accustomed to an unfavourable surrounding are used for immunising animals, the serum of which is now suitable for the treatment of human tuberculosis. It is too soon to speak of the effects of this line of treatment.

**Methods of Examination.**—(1) *Microscopic Examination.* Tuberculosis is one of the comparatively few diseases in which a diagnosis can usually be definitely made by microscopic examination alone. In the case of sputum, one of the yellowish fragments which are often present ought to be selected; dried films are then prepared in the usual way and stained by the Ziehl-Neelsen method (p. 101). In the case of urine or other fluids a deposit should first be obtained by centrifugalising a quantity in a test-tube, or by allowing the fluids to stand in a tall glass vessel (an ordinary burette is very convenient). Film preparations are then made with the deposit and treated as before. If a negative result is obtained in a suspected case, repeated examination should be undertaken. To avoid risk of contamination with the smegma bacillus the meatus of the urethra should be cleansed and the urine first passed should be rejected, or the urine may be drawn off with a sterile catheter. As stated above it is only exceptionally that difficulty will arise

to the experienced observer from this cause. (For points to be attended to, *vide* p. 255.)

(2) *Inoculation*.—The guinea-pig is the most suitable animal. If the material to be tested is a fluid it is injected subcutaneously or into the peritoneum; if solid or semi-solid it is placed in a small pocket in the skin, or it may be thoroughly broken up in sterile water or other fluid and the emulsion injected. By this method, material in which no tubercle bacilli can be found microscopically may sometimes be shown to be tubercular.

(3) *Cultivation*.—Owing to the difficulties this is usually quite impracticable as a means of diagnosis, and it is also unnecessary. The best method to obtain pure cultures is to produce tuberculosis in a guinea-pig by inoculation with tubercular material, and then, killing the animal after four or five weeks, to inoculate tubes of solidified blood serum, under strict aseptic precautions, with portions of a tubercular organ, *e.g.* the spleen. The portions of tissue should be fairly large, and should be well rubbed into the broken surface of the medium.

## CHAPTER X.

### LEPROSY.

LEPROSY is a disease of great interest, alike in its clinical and pathological aspects; whilst from the bacteriological point of view also, it presents some striking peculiarities. The invariable association of large numbers of characteristic bacilli with all leprous lesions is a well-established fact, and yet, so far, attempts to cultivate the bacilli outside the body, or to produce the disease experimentally in animals, have been attended with failure. Leprosy, so far as is known, is a disease which is confined to the human subject, but it has a very wide geographical distribution. It occurs in certain parts of Europe—Norway, Russia, Greece, etc., but is commonest in Asia, occurring in Syria, Persia, etc. It is prevalent in Africa, being especially found along the coast, in the Pacific Islands, in the warmer parts of North and South America, and also to a small extent in the northern part of North America. In all these various regions the disease presents the same general features, and the study of its pathological and bacteriological characters, wherever such has been carried on, has yielded similar results.

**Pathological Changes.**—Leprosy is characteristically a chronic disease, in which there is a great amount of tissue change, with comparatively little necessary impairment of the general health. In other words, the local effects of the bacilli are well marked, often extreme, whilst the toxic phenomena are proportionately at a minimum.

There are two chief forms of leprosy. The one, usually called the tubercular form—*lepra tuberosa* or *tuberculosa*—is characterised by the growth of granulation tissue in a nodular form or as a diffuse infiltration in the skin, in mucous membranes, etc., great disfigurement often resulting. In the other form, the anæsthetic,—maculo-anæsthetic of Hansen and Looft—the outstanding

changes are in the nerves, with consequent anæsthesia, paralysis of muscles, and trophic disturbances.

In the *tubercular* form the disease usually starts with the appearance of erythematous patches attended by a small amount of fever, and these are followed by the development of small nodular thickenings in the skin, especially of the face, of the backs of hands and feet, and of the extensor aspects of arms and

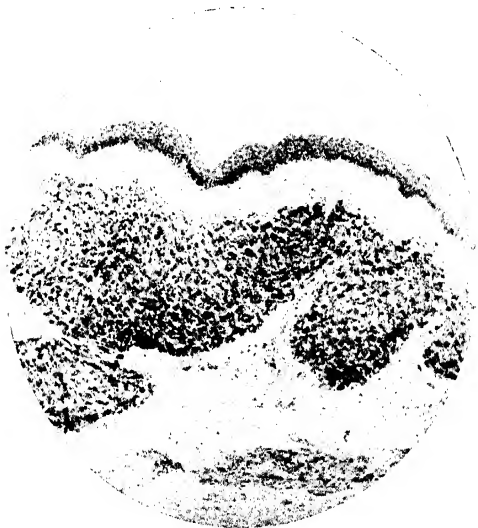


FIG. 91.—Sections through leprosy skin, showing the masses of cellular granulation tissue in the cutis ; the dark points are clumps of bacilli deeply stained.

Paraffin section ; Ziehl-Neelsen stain.  $\times 80$ .

legs. These nodules enlarge and produce great distortion of the surface, so that, in the case of the face, an appearance is produced which has been described as "leonine." The thickenings occur chiefly in the cutis (Fig. 91), to a less extent in the subcutaneous tissue. The epithelium often becomes stretched over them, and an oozing surface becomes developed, or actual ulceration may occur. The cornea and other parts of the eye, the mucous membrane of the mouth, larynx, and pharynx, may be the seat of similar nodular growths. Internal organs, especially the spleen, liver, and testicles, may become secondarily affected. In

all situations the change is of the same nature,—a chronic inflammatory condition attended by abundant formation of granulation tissue, nodular or diffuse in its arrangement. In this tissue a large proportion of the cells are of rounded or oval shape, like hyaline leucocytes; a number of these may be of comparatively large size, and may show vacuolation of their protoplasm and a vesicular type of nucleus. These are often known as “lepra-cells.” Amongst the cellular elements there is a varying amount of stroma, which in the earlier lesions is scanty and delicate, but in the older lesions may be very dense. Periarteritis is a common change, and very frequently the superficial nerves become involved in the nodules and undergo atrophy. The tissue in the leprous lesions is comparatively vascular, at least when young, and, unlike tubercular lesions, never shows caseation. Some of the lepra cells may contain several nuclei, but we do not meet with cells resembling in their appearance tubercle giant-cells, nor does an arrangement like that in tubercle follicles occur.

In the *anaesthetic* form the lesion of the nerves is the outstanding feature. These are the seat of diffuse infiltrations which lead to the destruction of the nerve fibres. In the earlier stages, in which the chief symptoms are pains along the nerves, there occur patches on the skin, often of considerable size, the margins of which show a somewhat livid congestion. Later, these patches become pale in the central parts, and the periphery becomes pigmented. There then follow remarkable series of trophic disturbances in which the skin, muscles, and bones are especially involved. The skin often becomes atrophied, parchment-like, and anaesthetic; frequently pemphigoid bullæ or other skin eruptions occur. Partly owing to injury to which the feet and arms are liable from their anaesthetic condition, and partly owing to trophic disturbances, necrosis and separation of parts are liable to occur. In this way great distortion results. The lesions in the nerves are of the same nature as those described above, that is, they are the result of a chronic inflammatory process, but the granulation tissue is scantier, and has a greater tendency to undergo cicatricial contraction. This is to be associated with the fact that the bacilli are present in fewer numbers.

**Bacillus of Leprosy.**—This bacillus was first observed in leprous tissues by Hansen in 1871, and was the subject of several communications by him in 1874 and later. Further researches, first by Neisser in 1879, and afterwards by observers in various parts of the world, agreed in their main results, and

confirmed the accuracy of Hansen's observations. The bacilli as seen in scrapings of ulcerated leprous nodules, or in sections, have the following characters. They are thin rods of practically the same size as tubercle bacilli, which they also resemble both in appearance and in staining reaction. They are straight or slightly curved, and usually occur singly, or two may be attached end to end; but they do not form chains. When stained they



FIG. 92.—Superficial part of leprous skin; the cells of the granulation tissue appear as dark patches, owing to the deeply-stained bacilli in their interior. In the upper part a process of epithelium is seen.

Paraffin section; stained with carbol-fuchsin and Bismarck-brown.  $\times 500$ .

may have a uniform appearance, or the protoplasm may be fragmented, so that they appear like short rows of cocci. They often appear tapered at one or both extremities; occasionally there is slight club-like swelling. Degenerated and partially broken down forms are also seen. They take up the basic aniline stains more readily than tubercle bacilli, but in order to stain them deeply a powerful stain, such as carbol-fuchsin, is necessary. When stained, they strongly resist decolorising, though they are more easily decolorised than tubercle bacilli. The best method is to stain with carbol-fuchsin as for tubercle

bacilli, but to use a weaker solution of sulphuric acid, say 5 per cent, in decolorising; in the case of films and thin sections, decolorising with such a solution for fifteen seconds is usually sufficient. Thereafter the tissues are coloured by a contrast stain, such as a watery solution of methylene-blue (*vide* p. 101). The bacilli are also readily stained by Gram's method. Regarding the presence of spores practically nothing is known, though some



FIG. 93.—High-power view of portion of leprosy nodule showing the arrangement of the bacilli within the cells of the granulation tissue.

Paraffin section; stained with carbol-fuchsin and methylene-blue.  $\times 1100$ .

of the unstained or stained points may be of this nature. We have, however, no means of testing their powers of resistance. Leprosy bacilli are non-motile.

**Position of the Bacilli.**—They occur in enormous numbers in the leprosy lesions, especially in the tubercular form. In fact, so numerous are they that the granulation tissue in sections, properly stained as above, presents quite a red colour under a low power of the microscope. The bacilli occur for the most part within the protoplasm of the round cells of the granulation tissue, and are often so numerous that the structure of the cells is quite



obscured (Fig. 92). They are often arranged in bundles which contain several bacilli lying parallel to one another, though the bundles lie in various directions (Fig. 93). The appearance thus presented by the cells filled with bacilli is very characteristic. Bacilli are also found free in the lymphatic spaces, but the greater number are undoubtedly contained within the cells. They are also found in spindle-shaped connective-tissue cells, in endothelial cells, and in the walls of blood vessels. They are for the most part confined to the connective tissue, but a few may be seen in the hair follicles and glands of the skin. Occasionally one or two may be found in the surface epithelium, where they probably have been carried by leucocytes, but this position is, on the whole, exceptional. They also occur in large numbers in the lymphatic glands associated with the affected parts. In the internal organs—liver, spleen, etc., when leprosy lesions are present, the bacilli are also found though in relatively smaller numbers. In the nerves in the anæsthetic form they are comparatively few, and in the sclerosed parts it may be impossible to find any. There are few also in the skin patches referred to above as occurring in this form of the disease.

Their spread is chiefly by the lymphatics, though distribution by the blood stream also occurs. They have been said to be found in the blood during the presence of fever and the eruption of fresh nodules, and they have also been observed in the blood vessels *post mortem*, chiefly contained within leucocytes. Recent observations (*e.g.* those of Doutrelepoint and Wolters) show that the bacilli may be more widely spread throughout the body than was formerly supposed. A few may be detected in some cases in various organs which show no structural change, especially in their capillaries. The brain and spinal cord are almost exempt, but in some cases bacilli have been found even within nerve cells.

**Relations to the Disease.**—Attempts to cultivate the leprosy bacilli outside the body have so far been unsuccessful. From time to time announcements of successful cultivations have been made, but one after another has proved to be erroneous. A similar statement may be made with regard to experiments on animals. If a piece of leprosy tissue be introduced subcutaneously in an animal, such as the rabbit, a certain amount of induration may take place around it, and the bacilli may be found unchanged in appearance weeks or even months afterwards, but no multiplication of the organisms occurs. The only exception to this statement is afforded by the experiments of Melcher and Orthmann, who inoculated the anterior chamber of the eye of rabbits with

leprous material, the inoculation being followed by an extensive growth of nodules in the lungs and internal organs, which they affirmed contained leprosy bacilli. It has been questioned, however, by several authorities whether the organisms in the nodules were really leprosy bacilli, and up to the present we cannot say that there is any satisfactory proof that the disease can be transmitted to any of the lower animals. Diphtheroid bacilli of more than one variety have been cultivated from the blood and tissues of leprous patients by Babes and others. Their presence would appear to be by no means infrequent, but it is not possible to say at present what their significance is.

It is interesting to note that a disease occurs under natural conditions in rats which presents many points of close similarity to leprosy. It has been observed in Russia, Germany, and England, and an excellent description has recently been given by Dean. In this affection there are lesions in the skin which resemble those in leprosy, and the cells contain enormous numbers of an acid-fast bacillus. The disease can be transmitted to rats by inoculation with the tissue juices containing the bacilli, but not to animals of other species. All attempts to cultivate the characteristic organism outside the body have failed, but Dean has obtained a diphtheroid bacillus—a result of interest in relation to what has been found in leprosy. Whether this disease has any relation to leprosy in the human subject is very doubtful, but the facts which have been ascertained may prove of high importance in connection with the pathology of the latter disease.

It would also appear that the disease is not readily inoculable in the human subject. In a well-known case described by Arning, a criminal in the Sandwich Islands was inoculated in several parts of the body with leprosy tissue. Two or three years later, well-marked tubercular leprosy appeared and led to a fatal result. This experiment, however, is open to the objection that the individual before inoculation had been exposed to infection in a natural way, having been frequently in contact with lepers. In other cases, inoculation experiments on healthy subjects and inoculations in other parts of leprous individuals have given negative results. It has been supposed by some that the failure to obtain cultures and to reproduce the disease experimentally may be partly due to the bacilli in the tissues being dead. That many of the leprous bacilli are in a dead condition is quite possible, in view of the long period during which dead tubercle bacilli introduced into the tissues of animals retain their form and staining reaction. There is also the fact that from time to time

in leprous subjects there occur febrile attacks, which are followed by a fresh outbreak of nodules, and it would appear that especially at these times multiplication of the bacilli takes place more actively.

The facts stated with regard to cultivation and inoculation experiments go to distinguish the leprosy bacillus all the more strongly from other organisms. Some have supposed that leprosy is a form of tubercle, or tubercle modified in some way, but for this there appears to us to be no evidence. Both from the pathological and from the bacteriological point of view the diseases are distinct. It should also be mentioned that tubercle is not uncommon complication in leprous subjects, in which case it presents the ordinary characters.

The mode by which leprosy is transmitted has been the subject of great controversy, and is one on which authorities still hold opposite opinions. Some consider that it is a hereditary disease, or at least that it is transmitted from a parent to the offspring; others again that it is transmitted by direct contact. There appears to be no doubt, however, that on the one hand leprous subjects may bear children free from leprosy, and that on the other hand, healthy individuals entering a leprous district may contract the disease, though this rarely occurs. Of the latter occurrence there is the well-known instance of Father Damien, who contracted leprosy after going to the Sandwich Islands. In view of all the facts there can be little doubt that leprosy in certain conditions may be transmitted by direct contact, though its contagiousness is not of a high order.

**Methods of Diagnosis.**—Film preparations should be made with the discharge from any ulcerated nodule which may be present, or from the scraping of a portion of excised tissue, and should be stained as above described. The presence of large numbers of bacilli situated within the cells and giving the staining reaction of leprosy bacilli, is conclusive. It is more satisfactory, however, to make microscopic sections through a portion of the excised tissue, when the structure of the nodule and the arrangement of the bacilli can be readily studied. The points of difference between leprosy and tubercle have already been stated, and in most cases there is really no difficulty in distinguishing the two conditions.

## CHAPTER XI.

### GLANDERS AND RHINOSCLEROMA.

#### GLANDERS.

THE bacillus of glanders (*bacillus mallei*; Fr., *bacille de la morve*; Ger., *Rotzbacillus*) was discovered by Löffler and Schutz, the announcement of this discovery being made towards the end of 1882. They not only obtained pure cultures of this organism from the tissues in the disease, but by experiments on horses and other animals conclusively established its causal relationship. These have been fully confirmed. The same organism has also been cultivated from the disease in the human subject, first by Weichselbaum in 1885, who obtained it from the pustules in a case of acute glanders in a woman, and by inoculation of animals obtained results similar to those of Löffler and Schütz.

Within recent years a substance, *mallein*, has been obtained from the cultures of the glanders bacillus by a method similar to that by which tuberculin was prepared, and has been found to produce corresponding effects in animals suffering from glanders to those produced by tuberculin in tuberculous animals.

**The Natural Disease.**—Glanders chiefly affects the equine species—horses, mules, and asses. Horned cattle, on the other hand, are quite immune, whilst goats and sheep occupy an intermediate position, the former being rather more susceptible and occasionally suffering from the natural disease. It also occurs in some of the carnivora—cats, lions, and tigers in menageries, which animals are infected from the carcases of animals affected with the disease. Many of the small rodents are highly susceptible to inoculation (*vide infra*).

Glanders is also found in man as the result of direct inoculation on some wound of the skin or other part by means of the discharges or diseased tissues of an animal affected, and hence is commonest amongst grooms and others whose work brings them

into contact with horses ; even amongst them it is a comparatively rare disease.

In horses the lesions are of two types, to which the names "glanders" proper and "farcy" have been given, though both may exist together. In glanders proper the septum nasi and adjacent parts are chiefly affected, there occurring in the mucous membrane nodules which are at first firm and of somewhat translucent grey appearance. The growth of these is attended usually by inflammatory swelling and profuse catarrhal discharge. Afterwards the nodules soften in the centre, break down, and give rise to irregular ulcerations. Similar lesions, though in less degree, may be found in the respiratory passages. Associated with these lesions there is usually implication of the lymphatic glands in the neck, mediastinum, etc. ; and there may be in the lungs, spleen, liver, etc., nodules of the size of a pea or larger, of greyish or yellow tint, firm or somewhat softened in the centre, and often surrounded by a congested zone. The term "farcy" is applied to the affection of the superficial lymphatic vessels and glands, which is specially seen where infection takes place through an abrasion of the skin, such as is often produced by the rubbing of the harness. The lymphatic vessels become irregularly thickened, so as to appear like knotted cords, and the associated lymphatic glands become enlarged and firm, though suppurative softening usually follows, and there may be ulceration. These thickenings are often spoken of as "farcy buds" and "farcy pipes." In farcy, also, secondary nodules may occur in internal organs and the nasal mucous membrane. The disease is often present in a "latent form," and its presence can only be detected by the mallein test (*vide infra*). In the ass the disease runs a more acute course than in the horse.

In man the disease is met with in two forms, an acute and a chronic ; though intermediate forms also occur, and chronic cases may take on the characters of the acute disease. The site of inoculation is usually on the hand or arm, by means of some scratch or abrasion, or possibly along a hair follicle, sometimes on the face, and occasionally on the mucous membrane of the mouth, nose, or eye. In the *acute* form there appears at the site of inoculation inflammatory swelling, attended usually with spreading redness, and the lymphatics in relation to the part also become inflamed, the appearances being those of a "poisoned wound." These local changes are soon followed by marked constitutional disturbance, and by an eruption on the surface of the body, at first papular and afterwards pustular, and later there may form in the subcutaneous tissue and muscles larger masses which soften and suppurate, the pus being often mixed with blood ; suppuration may occur also in the joints. In some cases the nasal mucous membrane may be secondarily infected, and thence inflammatory swelling may spread to the tissues of the face ; in others it remains free. The patient usually dies in two or three weeks, sometimes sooner, with the symptoms of rapid pyæmia. In

addition to the lesions mentioned there may be foci, usually suppurative, in the lungs (attended often with pneumonic consolidation), in the spleen, liver, bone-marrow, salivary glands, etc. In the *chronic* form the local lesion results in the formation of an irregular ulcer with thickened margins and sanious, often foul, discharge. The ulceration spreads deeply as well as superficially, and the thickened lymphatics also have a great tendency to ulcerate, though the lymphatic system is not so prominently affected as in the horse. Deposits may form in the subcutaneous tissue and muscles, and the mucous membrane may become affected. The disease may run a very chronic course, lasting for months, and recovery may occur, though, on the other hand, the disease may take on a more acute character and rapidly become fatal.

**The Glanders Bacillus.** — *Microscopical Characters.* — The glanders bacilli are minute rods, straight or slightly curved, with rounded ends, and about the same length as tubercle bacilli, but distinctly thicker (Fig. 94). They show, however, considerable variations in size and in appearance, and their pro-

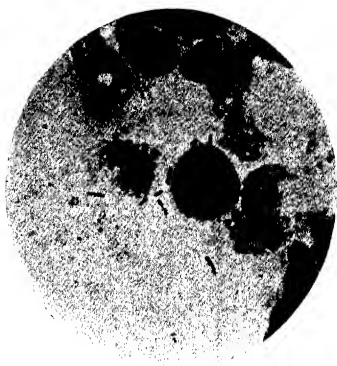


FIG. 94.—Glanders bacilli amongst broken-down cells. Film preparation from a glanders nodule in a guinea-pig. Stained with weak carbol-fuchsin.  $\times 1000$ .

toplasm is often broken up into a number of deeply-stained portions with unstained intervals between. These characters are seen both in the tissues and in cultures, but, as in the case of many organisms, irregularities in form and size are more pronounced in cultures (Fig 95); short filamentous forms 8 to 12  $\mu$  in length are sometimes met with, but these are on the whole rare. The organism is non-motile.

In the tissues the bacilli usually occur irregularly scattered amongst the cellular elements; a few may be contained within leucocytes and connective-tissue corpuscles, but the position of most is extracellular. They are most abundant in the acute lesions, in which they may be found in considerable numbers; but in the chronic nodules, especially when softening has taken place, they

are few in number, and it may be impossible to find any in sections. They have less powers of persistence, and disappear in the tissues much more quickly than tubercle bacilli.

There has been dispute as to whether or not they contain spores. Some consider certain of the unstained portions to be

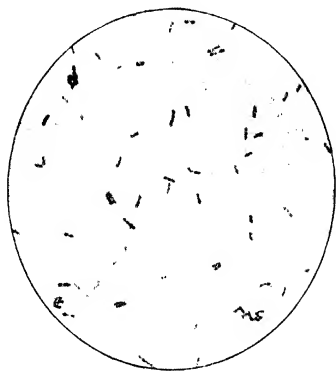


FIG. 95.—Glanders bacilli, from a pure culture on glycerin agar. Stained with carbol-fuchsin and partially decolorised to show segmentation of protoplasm.  $\times 1000$ .

of that nature, and it has been claimed that these can be stained by the method for staining spores (Rosenthal). But it is very doubtful that such is the case; the appearances correspond rather with mere breaks in the protoplasm, such as are met with in many other bacilli which do not contain spores, and the comparatively low powers of resistance of glanders bacilli containing these so-called spores, is strongly against their being of that nature. The power of resistance is after all the important practical point.

*Staining.*—The glanders bacillus differs widely from the tubercle bacillus in its staining reactions. It stains with simple watery solutions of the basic stains, but somewhat faintly (better when an alkali or a mordant, such as carbolic acid, is added), and even when deeply stained it readily loses the colour when a decolorising agent such as alcohol is applied. We have obtained the best results by carbol-thionin-blue (p. 98), and we prefer to dehydrate by the aniline-oil method. In film preparations of fresh glanders nodules the bacilli can be readily found by staining with any of the ordinary combinations, *e.g.* carbol-thionin-blue or weak carbol-fuchsin. By using a stain of suitable strength no decolorising agent is necessary, the film being simply washed in water, dried and mounted. M'Fadyean recommends that after sections have been stained in Löffler's methylene blue and slightly decolorised in weak acetic acid, they should be treated for fifteen minutes with a saturated solution of tannic acid; thereafter they are washed thoroughly in water, and as a contrast stain a 1 per cent solution of acid fuchsin may be applied for

half a minute ; they are then dehydrated, cleared, and mounted. Gram's method is quite inapplicable, the glanders bacilli rapidly losing the stain in the process.

**Cultivation.**—(For the methods of separation *vide infra*.) The glanders bacillus grows readily on most of the ordinary media, but a somewhat high temperature is necessary, growth taking place most rapidly at 35° to 37° C. Though a certain amount of growth occurs down to 21° C., a temperature above 25° C. is always desirable.

On *agar* and *glycerin agar* in stroke cultures growth appears along the line as a uniform streak of greyish-white colour and somewhat transparent appearance, with moist-looking surface, and when touched with a needle is found to be of rather slimy consistence. Later it spreads laterally for some distance, and the layer becomes of slightly brownish tint. On *serum* the growth is somewhat similar but more transparent, the separate colonies being in the form of round and almost clear drops. In sub-cultures on these media at the body temperature growth is visible within twenty-four hours, but when fresh cultures are made from the tissues it may not be visible till the second day. Serum or potato, however, is much more suitable for cultivating from the tissues than the agar media ; on the latter it is sometimes difficult to obtain growth.

In *broth*, growth forms at first a uniform turbidity, but soon settles to the bottom, and after a few days forms a pretty thick flocculent deposit of slimy and somewhat tenacious consistence.

On *potato* at 30° to 37° C. the glanders bacillus flourishes well and produces a characteristic appearance ; incubation at a high temperature, however, being necessary. Growth proceeds rapidly, and on the third day has usually formed a transparent layer of slightly yellowish tint, like clear honey in appearance. On subsequent days, the growth still extends and becomes darker in colour and more opaque, till about the eighth day it has a reddish-brown or chocolate tint, while the potato at the margin of the growth often shows a greenish-yellow staining. The characters of the growth on potato along with the microscopical appearances are quite sufficient to distinguish the glanders bacillus from every other known organism (sometimes the cholera organism and the *b. pyocyaneus* produce a somewhat similar appearance, but they can be readily distinguished by their other characters). Potato is also a suitable medium for starting cultures from the tissues ; in this case minute transparent colonies become visible on the third day and afterwards present the appearances just described.

**Powers of Resistance.**—The glanders bacillus is not killed at



once by drying, but usually loses its vitality after fourteen days in the dry condition, though sometimes it lives longer. It is not quickly destroyed by putrefaction, having been found to be still active after remaining two or three weeks in putrefying fluids. In cultures the bacilli retain their vitality for three or four months, if, after growth has taken place, they are kept at the temperature of the room; on the other hand, they are often found to be dead at the end of two weeks when kept constantly at the body temperature. They have comparatively feeble resistance to heat and antiseptics. Löffler found that they were killed in ten minutes in a fluid kept at 55° C., and in from two to three minutes by a 5 per cent solution of carbolic acid. Boiling water and the ordinarily used antiseptics are very rapid and efficient disinfectants.

We may summarise the characters of the glanders bacillus by saying that in its morphological characters it resembles somewhat the tubercle bacillus, but is thicker, and differs widely from it in its staining reactions. For its cultivation the higher temperatures are necessary, and the growth on potato presents most characteristic features.

**Experimental Inoculation.**—In horses subcutaneous injection of the glanders bacillus in pure culture reproduces all the important features of the disease. This fact was established at a comparatively early date by Löffler and Schutz, who, after one doubtful experiment, successfully inoculated two horses in this way, the cultures used having been grown for several generations outside the body. In a few days swellings formed at the sites of inoculation, and later broke down into unhealthy-looking ulcers. One of the animals died; after a few weeks the other, showing symptoms of cachexia, was killed. In both animals, in addition to ulcerations on the surface with involvement of the lymphatics, there were found, *post mortem*, nodules in the lungs, softened deposits in the muscles, and also affection of the nasal mucous membrane,—nodules, and irregular ulcerations. The ass is even more susceptible than the horse, the disease in the former running a more rapid course, but with similar lesions. The ass can be readily infected by simple scarification and inoculation with glanders secretion, etc. (Nocard).

Of small animals, field-mice and guinea-pigs are the most susceptible. Strangely enough, house-mice and white mice enjoy an almost complete immunity. In field-mice subcutaneous inoculation is followed by a very rapid disease, usually leading to death within eight days, the organisms becoming generalised and producing numerous minute nodules, especially in the spleen,

lungs, and liver. In the guinea-pig the disease is less acute, though secondary nodules in internal organs are usually present in considerable numbers. At the site of inoculation an inflammatory swelling forms, which soon softens and breaks down, leading to the formation of an irregular crateriform ulcer with indurated margins. The lymphatic vessels become infiltrated, and the corresponding lymphatic glands become enlarged to the size of peas or small nuts, softened, and semi-purulent. The animal sometimes dies in two or three weeks, sometimes not for a longer period. Secondary nodules, in varying numbers in different cases, may be present in the spleen, lungs, bones, nasal mucous membrane, testicles, ovaries, etc.; in some cases a few nodules are found in the spleen alone. Intraperitoneal injection in the male guinea-pig is followed, as pointed out by Straus, by a very rapid and semi-purulent affection of the tunica vaginalis, shown during life by great swelling and redness of the testicles, which changes may be noticeable in two or three days. By this method there occur also numerous small nodules on the surface of the peritoneum. Rabbits are less susceptible than guinea-pigs, and the effect of subcutaneous inoculation is somewhat uncertain. Accidental inoculation of the human subject with pure cultures of the bacillus has in more than one instance been followed by the acute form of the disease and a fatal result.

Mayer has found that when the glanders bacillus is injected along with melted butter into the peritoneum of a guinea-pig, it shows filamentous, branching, and club-shaped forms; in other words, it presents the characters of a streptothrix. Lubarsch, on the other hand, in a comparative study of the results of inoculation with acid-fast and other bacilli, found none of the above characters in the case of the glanders bacillus (*cf.* Tubercle).

**Action on the Tissues.**—From the above facts it will be seen that in many respects glanders presents an analogy to tubercle as regards the general characters of the lesions and the mode of spread. When the tissue changes in the two diseases are compared, certain differences are found. The glanders bacillus causes a more rapid and more marked inflammatory reaction. There is more leucocytic infiltration and less proliferative change which might lead to the formation of epithelioid cells. Thus the centre of an early glanders nodule shows a dense aggregation of leucocytes, most of which are polymorpho-nuclear, whilst in the central parts many show fragmentation of nuclei with the formation of a deeply staining granular detritus. And further, the inflammatory change may be followed by suppurative softening of the tissue, especially in certain situations, such as

the subcutaneous tissue and lymphatic glands. The nodules, therefore, in glanders, as Baumgarten puts it, occupy an intermediate position between miliary abscesses and tubercles. The diffuse coagulative necrosis and caseation which are so common in tubercle do not occur to the same degree in glanders, and typical giant cells are not formed. The nodules in the lungs show leucocytic infiltration and thickening of the alveolar walls, whilst the vesicles are filled with catarrhal cells; there may also be fibrinous exudation, whilst at the periphery of the nodules connective-tissue growth is present in proportion to their age. The tendency to spread by the lymphatics is always a well-marked feature, and when the bacilli gain entrance to the blood-stream, they soon settle in the various tissues and organs. Accordingly, even in acute cases it is usually quite impossible to detect the bacilli in the circulating blood, though sometimes they have been found. It is an interesting fact, shown by observations of the disease both in the human subject and in the horse, as well as by experiments on guinea-pigs, that the mucous membrane of the nose may become infected by means of the blood-stream—another example of the tendency of organisms to settle in special sites.

**Mode of Spread.**—Glanders usually spreads from a diseased animal by direct contagion with the discharge from the nose or from the sores, etc. So far as infection of the human subject goes, no other mode is known. There is no evidence that the disease is produced in man by inhalation of the bacilli in the dried condition. Some authorities consider that pulmonary glanders may be produced in this way in the horse, whilst others maintain that in all cases there is first a lesion of the nasal mucous membrane or of the skin surface, and that the lung is affected secondarily. Babes, however, found that the disease could be readily produced in susceptible animals by exposing them to an atmosphere in which cultures of the bacillus had been pulverised. He also found that inoculation of the skin with vaseline containing the bacilli might produce the disease, the bacilli in this case entering along the hair follicles.

**Agglutination of Glanders Bacilli.**—Shortly after the discovery of agglutination in typhoid fever, M'Fadyean showed that the serum of glandered horses possessed the power of agglutinating glanders bacilli. His later observations show that in the great majority of cases of glanders a 1 in 50 dilution of the serum produces marked agglutination in a few minutes, whilst in the great majority of non-glandered animals no effect is produced under these conditions. The test performed in the ordinary way is, however, not absolutely reliable, as exceptions occasionally occur in both directions, *i.e.* negative results by glandered animals and positive results by non-glandered animals. He finds that a more delicate

and reliable method is to grow the bacillus in bouillon containing a small proportion of the serum to be tested. In this way he has obtained a distinct sedimenting reaction with a serum which did not agglutinate at all distinctly in the ordinary method. Further observations are still required to determine the precise value of the test.

**Mallein and its Preparation.**—Mallein is obtained from cultures of the glanders bacillus grown for a suitable length of time, and, like tuberculin, is really a mixture comprising (1) substances in the bodies of the bacilli and (2) their soluble products, not destroyed by heat, along with substances derived from the medium of growth. It was at first obtained from cultures on solid media by extracting with glycerin or water, but is now usually prepared from cultures in glycerin bouillon. Such a culture, after being allowed to grow for three or four weeks, is sterilised by heat either in the autoclave at 115° C. or by steaming at 100° C. on successive days. It is then filtered through a Chamberland filter. The filtrate constitutes fluid mallein. Usually a little carbolic acid (·5 per cent) is added to prevent it from decomposing. Of such mallein 1 c.c. is usually the dose for a horse (M'Fadyean). Foth has prepared a dry form of mallein by throwing the filtrate of a broth culture, evaporated to one-tenth of its bulk, into twenty-five or thirty times its volume of alcohol. A white precipitate is formed, which is dried over calcium chloride and then under an air-pump. A dose of this dry mallein is ·05 to ·07 gm.

*The use of Mallein as a means of Diagnosis.*—In using mallein for the diagnosis of glanders, the temperature of the animal ought to be observed for some hours beforehand, and, after subcutaneous injection of a suitable dose, it is taken at definite intervals,—according to M'Fadyean at the sixth, tenth, fourteenth, and eighteenth hours afterwards, and on the next day. Here both the local reaction and the temperature are of importance. In a glandered animal, at the site of inoculation there is a somewhat painful local swelling, which reaches a diameter of five inches at least, the maximum size not being attained until twenty-four hours afterwards. The temperature rises 1·5° to 2° C., or more, the maximum generally occurring in from eight to sixteen hours. If the temperature never rises as much as 1·5°, the reaction is considered doubtful. In the negative reaction given by an animal free from glanders, the rise of temperature does not usually exceed 1°, the local swelling reaches the diameter of three inches at most, and has much diminished at the end of twenty-four hours. In the case of dry mallein, local reaction is less marked. Veterinary authorities are practically unanimous as to the great value of the mallein test as a means of diagnosis.

**Methods of Examination.**—Microscopic examination in a case of suspected glanders will at most reveal the presence of bacilli corresponding in their characters to the glanders bacillus. An absolute diagnosis cannot be made by this method. Cultures may be obtained by making successive strokes on blood serum or on glycerin agar (preferably the former), and incubating at 37° C. The colonies of the glanders bacillus do not appear till two days after. This method often fails unless a considerable number of the glanders bacilli are present. Another method is to dilute the secretion or pus with sterile water, to varying degrees, and then to smear the surface of potato with the mixture, the potatoes

being incubated at the above temperature. The colonies on potatoes may not appear till the third day. The most certain method, however, is by inoculation of a guinea-pig, either by subcutaneous or intraperitoneal injection. By the latter method, as above described, lesions are much more rapidly produced, and are more characteristic. If, however, there have been other organisms present, the animal may die of a septic peritonitis, though even in such a case the glanders bacilli will be found to be more numerous in the tunica vaginalis, and may be cultivated from this situation. It is extremely doubtful whether the application of mallein to diagnosis of the disease in the human subject is justifiable. There is a certain risk, that it may lead to the lesions assuming a more acute character; moreover, culture and inoculation tests are generally available. In the case of horses, etc., a diagnosis will, however, be much more easily and rapidly effected by means of mallein.

#### RHINOSCLEROMA.

This disease is considered here as, from the anatomical changes, it also belongs to the group of infective granulomata. It is characterised by the occurrence of chronic nodular thickenings in the skin or mucous membrane of the nose, or in the mucous membrane of the pharynx, larynx, or upper part of the trachea. The nodules are of considerable size, sometimes as large as a pea; in the earlier stages they are comparatively smooth on the surface, but later they become shrunken and the centre is often retracted. The disease is scarcely ever met with in this country, but is of not very uncommon occurrence on the Continent, especially in Austria and Poland. In the granulation tissue of the nodules there are to be found numerous round and rather large cells, which have peculiar characters and are often known as the cells of Mikulicz. Their protoplasm contains a collection of somewhat gelatinous material which may fill the cell and push the nucleus to the side. Within these cells there is present a characteristic bacillus, occurring in little clumps or masses chiefly in the gelatinous material. A few bacilli also lie free in the lymphatic spaces around. This organism was first observed by Frisch, and is now known as the bacillus of rhinoscleroma. The bacilli have the form of short oval rods, which, when lying separately, can be seen to possess a distinct capsule, and which in all their microscopical characters correspond closely with Friedländer's pneumobacillus. They are usually present in the lesions in a state of purity. It was at first stated

that they could be stained by Gram's method, but more recent observations show that like Friedländer's organism they lose the stain.

From the affected tissues this bacillus can be easily cultivated by the ordinary methods. In the characters of its growth in the various culture media it presents a close similarity to that of the pneumobacillus, as it also does in its fermentative action in milk and sugar-containing fluids. The nail-like appearance of the growth on gelatin is said to be less distinct, and the growth on potatoes is more transparent and may show small bubbles of gas; but it is doubtful whether any distinct line of difference can be drawn between the two organisms so far as their microscopical and cultural characters are concerned.

The evidence that the organisms described are the cause of this disease consists in their constant presence and their special relation to the affected tissues, as already described. From these facts alone it would appear probable that they are the active agents in the production of the lesions. Experimental inoculation has thrown little light on the subject, though one observer has described the production of nodules on the conjunctivæ of guinea-pigs. The relation of the rhinoscleroma organism to that of Friedländer is, however, still a matter of doubt, and the matter has been further complicated by the fact that a bacillus possessing closely similar characters has been found to be very frequently present in ozæna, and is often known as the *bacillus ozæne*. The last-mentioned organism is said to have more active fermentative powers. From what has been stated it will be seen that a number of organisms closely allied in their morphological characters, have been found in the nasal cavity in healthy or diseased conditions. There is no doubt that rhinoscleroma is a specific disease with well-marked characters and it is quite possible that one member of this group of organisms may be the causal agent, though indistinguishable from others by culture tests. There is, however, a tendency on the part of recent investigators to consider the "bacillus of rhinoscleroma" to be identical with the pneumobacillus and its presence in the affected tissues to represent merely a secondary invasion. The subject is one on which more light is still required.

## CHAPTER XII.

### ACTINOMYCOSIS AND ALLIED DISEASES.

ACTINOMYCOSIS is the most important example of a group of diseases produced by streptothrix organisms. It is related, by the characters of the pathological changes, to the diseases which have been described. The disease affects man in common with certain of the domestic animals, though it is more frequent in the latter, especially in oxen, swine, and horses. The parasite was first discovered in the ox by Bollinger, and described by him in 1877, the name *actinomyces* or *ray fungus* being from its appearance applied to it by the botanist Harz. In 1878 Israel described the parasite in the human subject, and in the following year Ponfick identified it as being the same as that found in the ox. Since that time a large number of cases have been observed in the human subject, the result of investigation being to show that it affects man much more frequently than was formerly supposed.

It is, however, to be noted that the term "actinomyces," as originally used, does not represent one parasite but a number of closely allied species, as cultures obtained from various sources have presented considerable differences; and, further, it is noted that other distinct species of streptothrix have been cultivated from isolated cases of disease in the human subject where the lesions resembled more or less closely those of actinomycosis. In one or two instances the organism has been found to be "acid-fast," and there is no doubt that the actinomyces group is closely related through intermediate forms with the tubercle group (*vide* p. 239).

**Naked-eye Characters of the Parasite.**—The actinomyces grows in the tissues in the form of little round masses or colonies, which, when fully developed, are easily visible to the naked eye, the largest being about the size of a small pin's head, whilst all sizes below this may be found. When suppuration is present,

they lie free in the pus ; when there is no suppuration, they are embedded in the granulation tissue, but are usually surrounded by a zone of softer issue. They may be transparent or jelly-like, or they may be opaque and of various colours—white, yellow, greenish, or almost black. The appearance depends upon their age and also upon their structure, the younger colonies being more or less transparent, the older ones being generally opaque. Their colour is modified by the presence of pigment and by degenerative change, which is usually accompanied by a yellowish coloration. They are generally of soft, sometimes tallow-like, consistence, though sometimes in the ox they are gritty, owing to the presence of calcareous deposit. They may be readily found in the pus by spreading it out in a thin layer on a glass slide and holding it up to the light. They are sometimes described as being always of a distinctly yellow colour, but this is only occasionally the case ; in fact, in the human subject they occur much more frequently as small specks of semi-translucent appearance, and of greenish-grey tint.

**Microscopical Characters.**—The parasite, which is now generally regarded as belonging to the streptothrix group of the higher bacteria (p. 14), presents pleomorphous characters. In the colonies, as they grow in the tissues, three morphological elements may be described, namely, filaments, coccus-like bodies, and clubs.

1. The *filaments* are comparatively thin, measuring about  $\cdot 6 \mu$  in diameter, but they are often of great length. They are composed of a central protoplasm enclosed by a sheath. The latter, which is most easily made out in the older filaments with granular protoplasm, occasionally contains granules of dark pigment. In the centre of the colony the filaments interlace with one another, and form an irregular network which may be loose or dense ; at the periphery they are often arranged in a somewhat radiating manner, and run outwards in a wavy or even spiral course. They also show true branching, a character which at once distinguishes them from the ordinary bacteria. Between the filaments there is a finely granular or homogeneous ground substance. Most of the colonies at an early stage are chiefly constituted by filaments loosely arranged ; but later, part of the growth may become so dense that its structure cannot be made out. This dense part, starting excentrically, may grow round the colony to form a hollow sphere, from the outer surface of which filaments radiate for a short distance (Fig. 96). The filaments usually stain uniformly in the younger colonies, but some, especially in the older colonies, may be segmented so



as to give the appearance of a chain of bacilli or of cocci, though the sheath enclosing them may generally be distinguished. Rod-shaped and spherical forms may also be seen lying free.

2. *Spores or Gonidia*.—Like other species of streptothrix the actinomyces when growing on a culture medium shows on its surface filaments growing upwards in the air, the protoplasm of which becomes segmented into rounded spores or gonidia. In natural conditions outside the body these gonidia become free

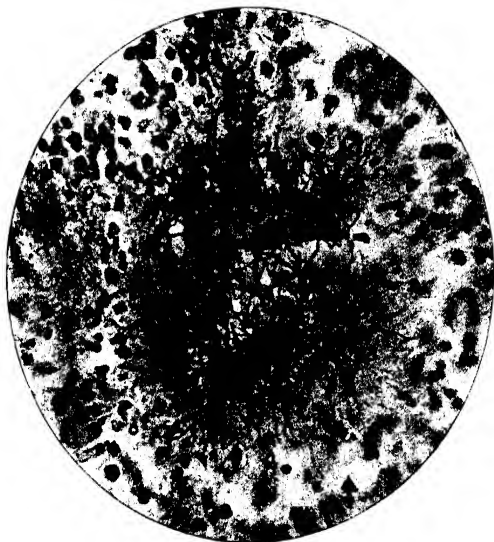


FIG. 96.—Actinomycosis of human liver, showing a colony of the parasite composed of a felted mass of filaments surrounded by pus.

Paraffin section ; stained by Gram's method and with safranin.  $\times 500$ .

and act as new centres by growing out into filaments. They have somewhat higher powers of resistance than the filaments, though less than the spores of most of the lower bacteria. An exposure to  $75^{\circ}$  C. for half an hour is sufficient to kill most streptothrices or their spores ; cultures containing spores can resist a temperature from five to ten degrees higher than spore-free cultures (Foulerton). It is probable that some of the spherical bodies formed within filaments when growing in the tissues have the same significance, *i.e.* are gonidia, whilst others may be merely the result of degenerative change. Both the

filaments and the spherical bodies are readily stained by Gram's method.

3. *Clubs*.—These are elongated pear-shaped bodies which are seen at the periphery of the colony, and are formed by a sort of hyaline swelling of the sheath around the free extremity of a filament (Figs. 97, 98). They are usually homogeneous and structureless in appearance. In the human subject the clubs are

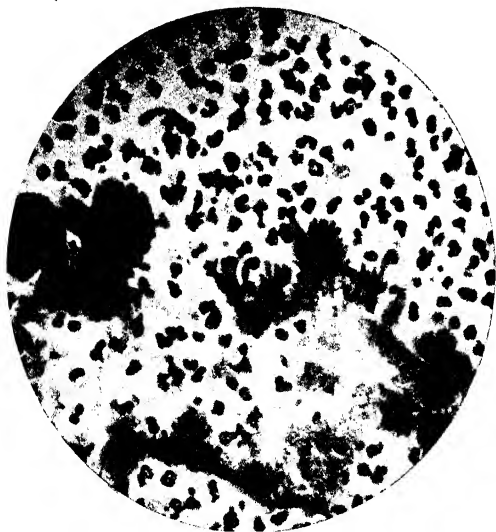


FIG. 97.—Actinomyces in human kidney, showing clubs radially arranged and surrounded by pus. The filaments had practically disappeared. Paraffin section; stained with hamatoxylin and rubin.  $\times 500$ .

often comparatively fragile structures, which are easily broken down, and may sometimes be dissolved in water. Sometimes they are well seen when examined in the fresh condition, but in hardened specimens are no longer distinguishable. In specimens stained by Gram's method they are usually not coloured by the violet, but take readily a contrast stain, such as picric acid, rubin, etc; sometimes a darkly-stained filament can be seen running for a distance in the centre, and may have a knob-like extremity. In many of the colonies in the human subject the clubs are absent. In the ox, on the other hand, where there are

much older colonies, the clubs constitute the most prominent feature, whilst in most colonies the filaments are more or less degenerated, and it may sometimes be impossible to find any. They often form a dense fringe around the colony, and when stained by Gram's method retain the violet stain. They have, in fact, undergone some further chemical change which produces the altered staining reaction. Occasionally in very chronic

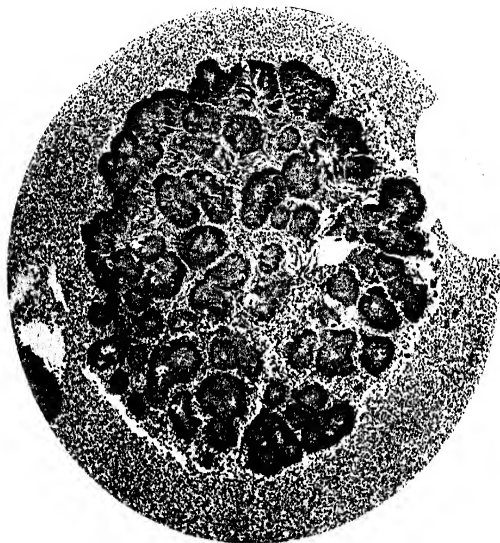


FIG. 98.—Colonies of actinomyces, showing general structural arrangement and clubs at periphery. From pus in human subject. Stained Gram and safranin.  $\times 60$ .

lesions in the human subject the clubs stain with Gram's method. Clubs showing intermediate staining reaction have been described in the ox by M'Fadyean. The club-formation probably represents a means of defence on the part of the parasite against the phagocytes of the tissue: the view, formerly held, that the clubs are organs of fructification has now been generally abandoned.

**Tissue Lesions.**—In the human subject the parasite produces by its growth a chronic inflammatory change, usually ending in a suppuration which slowly spreads. In some cases

there is a comparatively large production of granulation tissue, with only a little softening in the centre, so that the mass feels solid. This condition is sometimes found in the subcutaneous tissue, especially when the disease has not advanced far, and also in dense fibrous tissue. In most cases, however, and especially in internal organs, suppuration is the outstanding feature; this is associated with abundant growth of the parasite in the filamentous form presenting a honeycomb appearance. In an organ such as the liver, multiple foci of suppuration are seen at the spreading margin of the disease, whilst the colonies of the parasite may be seen in the pus with the naked eye. In the older parts the abscesses have become confluent, and formed large areas of suppuration. The pus is usually of greenish-yellow colour, and of somewhat slimy character.

In cattle the tissue reaction is more of a formative type, there being abundant growth of granulation tissue, which may result in large tumour-like masses, usually of more or less nodulated character, and often consisting of well-developed fibrous tissue containing areas of younger formation in which irregular abscess formation is usually present. The cells immediately around the colonies are usually irregularly rounded, or may even be somewhat columnar in shape, whilst farther out they become spindle-shaped and concentrically arranged. It is not uncommon to find leucocytes or granulation tissue invading the substance of the colonies, and portions of the parasite, etc., may be contained within leucocytes or within small giant-cells which are sometimes present. A similar invasion of old colonies by leucocytes is sometimes seen in human actinomycosis.

*Origin and Distribution of Lesions.*—The lesions in the human subject may occur in almost any part of the body, the paths of entrance being very various. In many cases the entrance takes place in the region of the mouth—probably around a decayed tooth—by the crypts of the tonsil, or by some abrasion. Swelling and suppuration may then follow in the vicinity and may spread in various directions. The periosteum of the jaw or the vertebrae may thus become affected, caries or necrosis resulting, or the pus may spread deeply in the tissues of the neck, and may even pass into the mediastinum. Occasionally the parasite may enter the tissues from the œsophagus, and in a considerable number of cases the primary lesion is in some part of the intestine, generally of the large intestine. The parasite penetrates the wall of the bowel, and may be found deeply between the coats, surrounded by purulent material. Thence it may spread to the peritoneum or to the extraperitoneal tissue,

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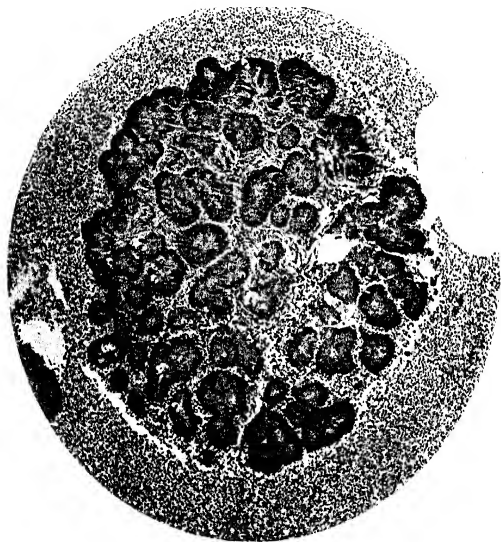


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the retrocæcal connective tissue and that around the rectum being not uncommonly seats of suppuration produced in this way. A peculiar affection of the intestine has been described, in which slightly raised plaques are found both in the large and small intestines, these plaques being composed almost exclusively of masses of the actinomyces along with epithelial cells. This, however, is a rare condition. The path of entrance may also be by the respiratory passages, the primary lesion being pulmonary or peribronchial; extensive suppuration in the lungs may result. Infection may also occur by the skin surface, and lastly, by the female genital tract, as in a case recorded by Grainger Stewart and Muir, in which both ovaries and both Fallopian tubes were affected.

When the parasite has invaded the tissues by any of these channels, secondary or "metastatic" abscesses may occur in internal organs. The liver is the organ most frequently affected, though abscesses may occur in the lungs, brain, kidneys, etc. In such cases the spread takes place by the blood stream, and it is possible that leucocytes may be the carriers of the infection, as it is not uncommon to find leucocytes in the neighbourhood of a colony containing small portions of the filaments in their interior.

In the ox, on the other hand, the disease usually remains quite local, or spreads by continuity. It may produce tumour-like masses in the region of the jaw or neck, or it may specially affect the palate or tongue, in the latter producing enlargement and induration, with nodular thickening on the surface—the condition known as "woody tongue."

*Source of the Parasite.*—There is a considerable amount of evidence to show that outside the body the parasite grows on grain, especially on barley. Both in the ox and in the pig the parasite has been found growing around fragments of grain embedded in the tissues. There are besides, in the case of the human subject, a certain number of cases in which there was a history of penetration of a mucous surface by a portion of grain, and in a considerable proportion of cases the patient has been exposed to infection from this source. The position of the lesions in cattle is also in favour of such a view.

**Cultivation** (for methods of isolation see later).—The descriptions of the cultures obtained by various investigators differ in essential particulars, and there is no doubt that the organisms described are different. The following is the account of the organism as cultivated by Boström:—

On *agar* or *glycerin agar* at 37° C., growth is generally

visible on the third or fourth day in the form of little transparent drops which gradually enlarge and form rounded projections of a reddish-yellow tint and somewhat transparent appearance, like drops of amber. The growths tend to remain separate, and even when they become confluent, the nodular character is maintained. They have a tough consistence, being with difficulty broken up, and adhere firmly to the surface of the agar. Older growths often show on the surface a sort of corrugated aspect, and may sometimes present the appearance of having been dusted with a brownish-yellow powder (Fig. 99).

In the cultures at an early stage the growth is composed of branching filaments, which stain uniformly (Fig. 100), but later some of the superficial filaments may show segmentation into gonidia. Slight bulbous thickenings may be seen at the end of some of the filaments, but true clubs have not been observed.

On *gelatin* the same tendency to grow in little spherical masses is seen, and the medium becomes very slowly liquefied.

When this occurs the liquefied portion has a brownish colour and somewhat syrupy consistence, and the growths may be seen at the bottom, as little balls, from the surface of which filaments radiate.

The organism obtained in culture by Wolff and Israel (*vide infra*) is probably the same as the one which has been recently described in detail by J. H. Wright, who obtained it in pure condition from fifteen different cases of the disease. It differs markedly from Boström's organism in being almost a strict anaerobe and in



FIG. 99.—Cultures of the actinomycetes on glycerin agar, of about three weeks' growth, showing the appearances which occur. The growth in A is at places somewhat corrugated on the surface. Natural size.



ceasing to grow at a temperature a little below that of the body. Under ordinary aerobic conditions either no growth occurs or it is of a very slight character. On the surface of agar under anaerobic conditions the organism produces dense rounded colonies of greyish-white colour, which sometimes assume a rosette form. A somewhat curious feature of growth is described by Wright, namely, that in a shake culture in glucose agar the colonies are most numerous, and form a dense zone about half an inch from the surface of the medium, that is, at a level

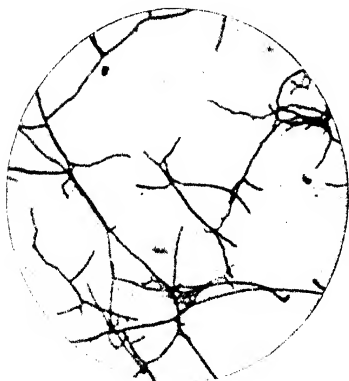


FIG. 100.—Actinomyces, from a culture on glycerin agar, showing the branching of the filaments.

Stained with fuchsin.  $\times 1000$ .

where there is presumably a mere trace of oxygen obtainable (Fig. 101). In bouillon, growth takes place at the bottom of the medium in rounded masses which afterwards undergo disintegration. Wright found when the organism was grown in the presence of serum or other animal fluids that the formation of true clubs occurred at the extremity of some of the filaments (Fig. 102). From the conditions under which growth occurs, he is inclined to regard it as a true parasite, and doubts whether it

can have a saprophytic existence outside the body, *e.g.* on grain. He is also of opinion that all cases of true actinomycosis, *i.e.* cases where colonies visible to the naked eye are present, are probably produced by one species, and that the aerobic organisms obtained by Boström and others are probably accidental contaminations. It is quite evident that further investigations are required in the light of the results detailed. Certainly the parasite in many cases of actinomycosis in the human subject does not grow on ordinary media under aerobic conditions as Boström's organism does.

**Varieties of Actinomyces and Allied Forms.**—It is probable that in the cases of the disease described in the human subject there is more than one variety or species of parasite belonging to the same group. Gasperini has described several varieties of *actinomyces bovis* according

to the colour of the growths, and a similar condition may obtain in the case of the human subject. Furthermore a considerable number of *streptothrices* have been found in cases of disease in the human subject, the associated lesions varying in character from tubercle-like nodules on the one hand to suppurative processes on the other. The organisms cultivated from such sources differ according to their microscopic characters (for example, some form "clubs" whilst others do not) according to their conditions of growth, staining reactions, etc. Of these only a few examples may here be mentioned, but it may be noted that the importance of the streptothrices as causes of disease is constantly being extended. Wolff and Israel cultivated from two cases of "actinomycosis" in man a streptothrix which differs in so many important points from the actinomyces of Boström



FIG. 101.<sup>1</sup>—Shake cultures of actinomyces in glucose agar, showing the maximum growth at some distance from the surface of the medium.



FIG. 102.—Section of a colony of actinomyces from a culture in blood serum, showing the formation of clubs at the periphery.  $\times 1500$ .

that it is now regarded as a distinct species. Another species was cultivated by Eppinger from a brain abscess, and called by him "*cladothrixasteroides*," from the appearance of its colonies on culture media. A case of general streptothrix infection in the human subject described by MacDonald was probably due to the same organism as Eppinger's. In the tissues it grows in a somewhat diffuse manner and does not form clubs;

<sup>1</sup> For Figs. 101 and 102 we are indebted to Dr. J. Homer Wright of Boston, U.S.A.

in rabbits and guinea-pigs it produces tubercle-like lesions. Flexner observed a streptothrix in the lungs associated with lesions somewhat like a rapid phthisis, and applied the name "pseudo-tuberculosis hominis streptothricea"; an apparently similar condition has been described by Buchholz. Berestnew cultivated two species of streptothrix from suppurative lesions, one of which is acid-fast and grows only in anaerobic conditions. Birt and Leishman have described another acid-fast streptothrix obtained from cirrhotic nodules in the lungs of a man. This organism grows readily on ordinary media, forming a white powdery growth which afterwards assumes a pinkish colour; it is pathogenic for guinea-pigs, in which it causes caseous lesions. There is, further, the streptothrix *madura* described below.

In diseases of the lower animals several other forms have been found. For example, a streptothrix has been shown by Nocard to be the cause of a disease of the ox,—"*farcin du bœuf*,"—a disease in which also there occur tumour-like masses of granulation tissue. Dean has cultivated from a nodule in a horse another streptothrix, which produces tubercle-like nodules in the rabbit with club-formation; it has close resemblances to the organism of Israel and Wolff. The so-called diphtheria of calves and "bacillary necrosis" in the ox are probably both produced by another streptothrix or leptothrix, which grows diffusely in the tissues in the form of fine felted filaments. Further investigation may show that some of these or other species may occur in the human subject in conditions which are not yet differentiated.

**Experimental Inoculation.**—Inoculation of smaller animals, such as rabbits and guinea-pigs, has usually failed to give positive results. This was the case, for example, in the important series of experiments by Boström, and it may be assumed that these animals are little susceptible to the actinomycetes. The disease has, however, been experimentally produced in the bovine species both by cultures from the ox and from the human subject. Inoculation with the organism of Israel and Wolff produces nodular lesions both in rabbits and in guinea-pigs, while Wright found that characteristic colonies and lesions resulted although the parasite did not grow to any great extent. Several of the other species of streptothrix have been found to possess active pathogenic properties.

**Methods of Examination and Diagnosis.**—As actinomycosis cannot be diagnosed with certainty apart from the discovery of the parasite, a careful examination of the pus in obscure cases of suppuration should always be undertaken. As already stated, the colonies can be recognised with the naked eye, especially when some of the pus is spread out on a piece of glass. If one of these is washed in salt solution and examined unstained, the clubs, if present, are at once seen on microscopic examination. Or the colony may be stained with a simple reagent such as picrocarmine, and mounted in glycerin or Farrant's solution. To study the filaments, a colony should be broken down on a

cover-glass, dried, and stained with a simple solution of any of the basic aniline dyes, such as gentian-violet, though better results are obtained by carbol-thionin-blue, or by carbol-fuchsin diluted with five parts of water. If the specimen be over-stained, it may be decolorised by weak acetic acid. Cover-glass preparations of this kind, and also of cultures, are readily stained by these methods, but in the case of sections of the tissues Gram's method, or a modification of it, should be used to show the filaments, etc., a watery solution of acid fuchsin being afterwards used to stain the clubs. By this method, very striking preparations may be obtained.

Cultures should be made both under aerobic and anaerobic conditions. Tubes of agar or glycerin agar should be inoculated and incubated at 37° C.; deep tubes of melted glucose agar should also be used, the inoculated material being diffused through the medium, separate colonies may thus be obtained. Owing to the slow growth of the actinomyces, however, the obtaining of pure cultures is difficult, unless the pus is free from contamination with other organisms.

#### MADURA DISEASE.

Madura disease or mycetoma resembles actinomycosis both as regards the general characters of the lesions and the occurrence of the parasite in the form of colonies or "granules." There is no doubt, however, that the two conditions are distinct, and it also appears established that the two varieties of Madura disease (*vide infra*) are produced by different organisms. This disease is comparatively common in India and in various other parts of the tropics: it has also been met with in Algiers and in America. Madura disease differs from actinomyces not only in its geographical distribution but also in its clinical characters. Its course, for example, is of an extremely chronic nature, and though the local disease is incurable except by operation, the parasite never produces secondary lesions in internal organs. Vincent also found that iodide of potassium, which has a high value as a therapeutic agent in many cases of actinomycosis, had no effect in the case of Madura disease studied by him. It most frequently affects the foot; hence the disease is often spoken of as "Madura foot." The hand is rarely affected. In the parts affected there is a slow growth of granulation tissue which has an irregularly nodular character, and in the centre of the nodules there occurs purulent softening which is often followed by the

formation of fistulous openings and ulcers. There are great enlargement and distortion of the part and frequently caries and necrosis of the bones. Within the softened cavities and also in the spaces between the fibrous tissue, small rounded bodies or granules, bearing a certain resemblance to the actinomyces, are present. These may have a yellowish or pinkish colour, compared from their appearance to fish roe, or they may be black like grains of gunpowder, and may by their conglomeration form nodules of considerable size. Hence a *pale variety* and a *black variety* of the disease have been distinguished; in both varieties the granules mentioned reach a rather larger size

than in actinomycosis. These two conditions will be considered separately.

**Pale Variety.**—When the roe-like granules are examined microscopically, they are found, like the actinomyces, to show in their interior an abundant mass of branching filaments with mycelial arrangement. There may also be present at the periphery club-like structures, as in actinomyces; sometimes they are absent. These structures often have an elongated wedge-shape, forming an outer zone to the colony, and in some cases the



FIG. 103. — *Streptothrix Maduræ*, showing branching filaments. From a culture on agar. Stained with carbol-thionin-blue.  $\times 1000$ .

filaments can be found to be connected with them. Vincent obtained cultures of the parasite from a case in Algiers, and found it to be a distinct species: it is now known as the *streptothrix Maduræ*. Morphologically it closely resembles the actinomyces, but it presents certain differences in cultural characters. In gelatin it forms raised colonies of a yellowish colour, with umbilication of the centre, and there is no liquefaction of the medium. On agar the growth assumes a reddish colour; the organism flourishes well in various vegetable infusions in which the actinomyces does not grow. On all the media growth only takes place in aerobic conditions. Experimental inoculation of various animals has failed to reproduce the disease. There is

therefore no doubt that the *streptothrix maduræ* and the *actinomyces* are distinct species.

**Black Variety.**—The observations of J. H. Wright, who obtained pure cultures of a hyphomycete, show that this variety is a distinct affection from the pale variety. The pigment may be dissolved by soaking the granules for a few minutes in hypochlorite of sodium solution, and the granules may then be crushed out beneath a cover-glass and examined microscopically. The black granules are composed of a somewhat homogeneous ground-substance impregnated with pigment, and in this there is a mycelium of thick filaments or hyphæ, many of the segments of which are swollen; at the periphery the hyphæ form a zone with radiate arrangement. In many of the older granules the parasite is largely degenerated and presents an amorphous appearance. Wright planted over sixty of the black granules in various culture media, and obtained cultures of a hyphomycete from about a third of these. The organism grows well on agar, bouillon, potato, etc.; on agar it forms a felted mass of greyish colour, and in old cultures black granules appear amongst the mycelium. Microscopically the parasite appears as a mycelium of thick branching filaments with delicate transverse septa; in the older threads the segments become swollen, so that strings of oval-shaped bodies result. No signs of spore-formation were noted. Inoculation of animals with cultures gave negative results, as did also direct inoculation with the black granules from the tissues.

## CHAPTER XIII

### ANTHRAX.<sup>1</sup>

*OTHER NAMES.*—SPLENIC FEVER, MALIGNANT PUSTULE, WOOL-SORTER'S DISEASE. *GERMAN*, MILZBRAND; *FRENCH*, CHARBON.<sup>2</sup>

**Introductory.**—Anthrax is a disease occurring epidemically among the herbivora, especially sheep and oxen, in which animals it has the characters of a rapidly fatal form of septicæmia with splenic enlargement, attended by an extensive multiplication of characteristic bacilli throughout the blood. The disease does not occur as a natural affection in man, but may be communicated to him directly or indirectly from animals, and it may then appear in certainly two and possibly three forms. In the first there is infection through the skin, in which a local lesion, the "malignant pustule," occurs. In the second form infection takes place through the respiratory tract. Here very aggravated symptoms centred in the thorax, with rapidly fatal termination, follow. Thirdly, an infection may probably take place through the intestinal tract, which is now the first part to give rise to symptoms. In all these forms of the affection in the human subject, the bacilli are in their distribution much more restricted to the local lesions than is the case in the ox, their growth and spread being attended by inflammatory œdema and often by hæmorrhages.

**Historical Summary.**—Historical researches leave little doubt that from the earliest times anthrax has occurred among cattle. For a long time its pathology was not understood, and it went by many names.

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<sup>1</sup> In even recent works on surgery the term "anthrax" may be found applied to any form of carbuncle. Before its true pathology was known the local variety of the disease which occurs in man and which is now called "malignant pustule" was known as "malignant carbuncle."

<sup>2</sup> This must be distinguished from "charbon symptomatique," which is quite a different disease.

During the early part of last century much attention was paid to it, and, with a view to finding out its nature and means of spread, various conditions attaching to its occurrence, such as those of soil and weather, were exhaustively studied. Pollender in 1849 pointed out that the blood of anthrax animals contained numerous rod-shaped bodies which he conjectured had some causal connection with the disease. In 1863 Davaine announced that they were bacteria, and originated the name *bacillus anthracis*. He stated that unless blood used in inoculation experiments on animals contained them, death did not ensue. Though this conclusion was disputed, still by the work of Davaine and others the causal relationship of the bacilli to the disease had been nearly established when the work of Koch appeared in 1876. This constituted that observer's first contribution to bacteriology, and did much to clear up the whole subject. Koch confirmed Davaine's view that the bodies were bacteria. He observed in the blood of anthrax animals the appearance of division, and from this deduced that multiplication took place in the tissues. He observed them under the microscope dividing outside the body, and noticed spore formation taking place. He also isolated the bacilli in pure culture outside the body, and, by inoculating animals with them, produced the disease artificially. In his earlier experiments he failed to produce death by feeding susceptible animals both with bacilli and spores, and as the intestinal tract was, in his view, the natural path of infection, he considered as incomplete the proof of this method of the spontaneous occurrence of anthrax in herds of animals. Koch's observations were, shortly afterwards, confirmed in the main by Pasteur, though controversy arose between them on certain minor points. Moreover, further research showed that the disease could be produced in animals by feeding them with spores, and thus the way in which the disease might spread naturally was explained.

**The Bacillus Anthracis.**—Anthrax as a disease in man is of comparative rarity. Not only, however, is the bacillus anthracis easy of growth and recognition, but in its growth it illustrates many of the general morphological characters of the whole group of bacilli, and is therefore of the greatest use to the student. Further, its behaviour when inoculated in animals illustrates many of the points raised in connection with such difficult questions as the general pathogenic effects of bacteria, immunity, etc. Hence an enormous amount of work has been done in investigating it in all its aspects.

If a drop of blood is taken immediately after death from an auricular vein of a cow, for example, which has died from anthrax, and examined microscopically, it will be found to contain a great number of large non-motile bacilli. On making a cover-glass preparation from the same source, and staining with watery methylene-blue, the characters of the bacilli can be better made out. They are about  $1.2\ \mu$  thick or a little thicker, and 6 to  $8\ \mu$  long, though both shorter and longer forms also occur. The ends are sharply cut across, or may be slightly dimpled so as to resemble somewhat the proximal end of a phalanx. Their



protoplasm is very finely granular, and sometimes appears surrounded by a thin unstained capsule. When several bacilli lie end to end in a thread, the capsule seems common to the whole thread (Fig. 108). They stain well with all the basic aniline dyes and are not decolorised by Gram's method.

*Plate Cultures.*—From a source such as that indicated, it is easy to isolate the bacilli by making gelatin or agar plates. If, after twelve hours' incubation at 37° C., the latter be examined under a low objective, colonies will be observed. They are to be recognised by beautiful wavy wreaths like locks of hair, radiating from the centre and apparently terminating in a point

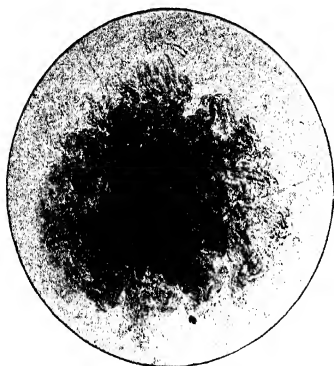


FIG. 104.—Surface colony of the anthrax bacillus on an agar plate, showing the characteristic appearances.  $\times 30$ .

which, however, on examination with a higher power is observed to be a filament which turns upon itself (Fig. 104). The whole colony is, in fact, probably one long thread. Such colonies are very suitable for making impression preparations (*vide* p. 118) which preserve permanently the appearances described. On examining such with a high power, the wreaths are seen to be made up of bundles of long filaments lying parallel with one another, each filament consisting of a chain of bacilli lying end

to end, and similar to those observed in the blood (Fig. 105).

On gelatin plates, after from twenty-four to thirty-six hours at 20° C., the same appearances manifest themselves, and later they are accompanied by liquefaction of the gelatin. In gelatin plates, however, instead of the characteristically wreathed appearance at the margin, the colonies sometimes give off radiating spikelets irregularly nodulated, which produce a star-like form. These spikelets are composed of spirally twisted threads.

From such plates the bacilli can be easily isolated, and the appearances of pure cultures on various media studied.

In *bouillon*, after twenty-four hours' incubation at 37° C., there is usually the appearance of irregularly spiral threads suspended in the liquid. These, on being examined, are seen

to be made up of bundles of parallel chains of bacilli. Later, growth is more abundant, and forms a flocculent mass at the bottom of the fluid.

In *gelatin* stab cultures, the characteristic appearance can be best observed when a low proportion, say  $7\frac{1}{2}$  per cent, of gelatin

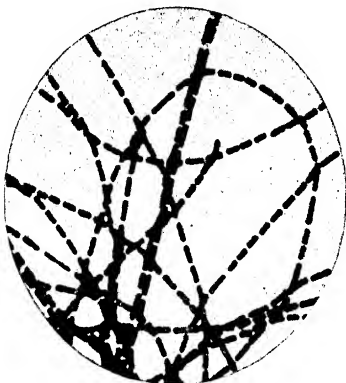


FIG. 105.—Anthrax bacilli, arranged in chains, from a twenty-four hours' culture on agar at  $37^{\circ}$  C.

Stained with fuchsin.  $\times 1000$ .

is present, and when the tube is directly inoculated from anthrax blood. In about two days there radiate out into the medium from the needle track numberless very fine spikelets which enable the cultures to be easily recognised. These spikelets are longest at the upper part of the needle track (Fig. 106). Not much spread takes place on the surface of the gelatin, but here liquefaction commences, and gradually spreads down the stab and out into the medium, till the whole of the gelatin may be liquefied. Gelatin slope cultures exhibit a thick felted growth, the edges of which show the wreathed appearance seen in plate cultures. Liquefaction here soon ploughs a trough in the surface of the medium. Sometimes "spiking" does not take place in gelatin stab cultures, only



FIG. 106.—Stab culture of the anthrax bacillus in peptone-gelatin; seven days' growth. It shows the "spiking" and also, at the surface, commencing liquefaction. Natural size.

little round particles of growth occurring down the needle track, followed by liquefaction. As has been shown by Richard Muir, this property of spiking can be restored by growing the bacillus for twenty-four hours on blood agar at 37° C. Agar sloped cultures have the appearance of similar cultures in gelatin, though, of course, no liquefaction takes place.

*Blood serum* sloped cultures present the same appearances as those on agar. The margin of the surface growth on any of the solid media shows the characteristic wreathing seen in plate colonies.

On *potatoes* there occurs a thick felted white mass of bacilli showing no special characters. Such a growth, however, is useful for studying sporulation.

The anthrax bacillus will thus grow readily on any of the ordinary media. It can usually be sufficiently recognised by its microscopic appearance, by its growth on agar or gelatin plates, and by its growth in gelatin stab cultures. The growth on plates is specially characteristic, and is simulated by no other pathogenic organism.

**The Biology of the B. Anthracis.**—Koch found that the bacillus anthracis grows best at a temperature of 35° C. Growth, *i.e.* multiplication, does not take place below 12° C. or above 45° C. In the spore-free condition the bacilli have comparatively low powers of resistance. They do not stand long exposure to 60° C., and if kept at ordinary temperature in the dry condition they are usually found to be dead after a few days. The action of the gastric juice is rapidly fatal to them, and they are accordingly destroyed in the stomachs of healthy animals. They are also soon killed in the process of putrefaction. They can, however, be cooled below the freezing-point without dying. The bacillus can grow without oxygen, but some of its vital functions are best carried on in the presence of this gas. Thus in anthrax cultures the liquefaction of gelatin always commences at the surface and spreads downwards. Growth is more rapid in the presence of oxygen, and spore formation does not occur in its absence. The organism may be classed as a facultative anaerobe.

*Sporulation.*—Under certain circumstances sporulation occurs in anthrax bacilli. The morphological appearances are of the ordinary kind. A little highly refractile speck appears in the protoplasm about the centre of the bacillus; this gradually increases in size until it forms an oval body about the same thickness as the bacillus lying in the bacillary protoplasm (Fig. 107). The latter gradually loses its staining capacities and finally disappears. The spore thus lies free as an oval highly

refractile body which does not stain by ordinary methods, but which can be easily stained by the special methods described for such a purpose (p. 102). When the spore is again about to assume the bacillary form the capsule is apparently absorbed, and the protoplasm within grows out, taking on the ordinary rod-shaped form.

According to most observers sporulation never occurs within the body of an animal suffering from anthrax. Koch attributes this, probably rightly, to the absence of free oxygen. The latter gas he found necessary to the occurrence of spores in cultures outside the body. Many, however, are inclined to assign as the cause of sporulation the absence of the optimum pabulum, which in the case of anthrax is afforded by the animal tissues. Besides these conditions there is another factor necessary to sporulation, viz. a suitable temperature. The optimum temperature for spore production is 30° C. Koch found that spore formation did not occur below 18° C. Above 42° C. not only does sporulation cease, but Pasteur found that if bacilli were kept at this temperature for eight days they did not regain the capacity when again grown at a lower temperature. In order to make them again capable of sporing it is necessary to adopt special measures, such as passage through the bodies of a series of susceptible animals.

Anthrax spores have extremely high powers of resistance. In a dry condition they will remain viable for a year or more. Koch found they resisted boiling for five minutes; and dry heat at 140° C. must be applied for several hours to kill them with certainty. Unlike the bacilli, they can resist the action of the gastric juice for a long period of time. They are often used as test objects by which the action of germicides is judged. For this purpose an emulsion is made by scraping off a surface culture and rubbing it up in a little sterile water. Into this

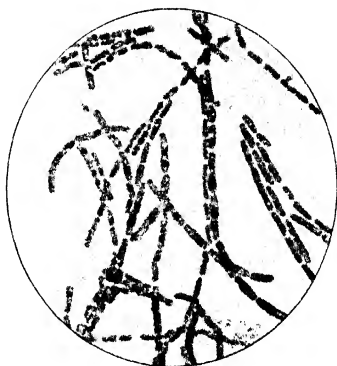


FIG. 107.—Anthrax bacilli containing spores (the darkly coloured bodies); from a three days' culture on agar at 37° C. Stained with carbol-fuchsin and methylene-blue.  $\times 1000$ .

sterile silk threads are dipped, which, after being dried over strong sulphuric acid in a desiccator, can be kept for long periods of time in an unchanged condition. For use they are placed in the germicidal solution for the desired time, then washed with water to remove the last traces of the reagent and laid on the surface of agar or placed in bouillon, in order that if death has not occurred growth may be observed (*see* Chap. IV.).

**Anthrax in Animals.**—Anthrax occurs from time to time epidemically in sheep, cattle, and, more rarely, in horses and deer. These epidemics are found in various parts of the world, although they are naturally most far-reaching where legal precautions to prevent the spread of infection are non-existent. All the countries of Europe are from time to time visited by the disease, but in some it is much more common than in others. In Britain the death-rate is small, but in France the annual mortality among sheep was probably 10 per cent of the total number in the country, and among cattle 5 per cent. These figures, however, have been largely modified by the system of preventive treatment which will be presently described. In sheep and cattle the disease is specially virulent. An animal may suddenly drop down, with symptoms of collapse, quickening of pulse and respiration, and dyspnoea, and death may occur in a few minutes. In less acute cases the animal is apparently out of sorts, and does not feed; its pulse and respiration are quickened; rigors occur, succeeded by high temperature; there is a sanguineous discharge from the bowels, and bloody mucus may be observed about the mouth and nose. There may be convulsive movements, there is progressive weakness, with cyanosis, death occurring in from twelve to forty-eight hours. In the more prolonged cases widespread oedema and extensive enlargement of lymphatic glands are marked features; and in the glands, especially about the neck, actual necrosis with ulceration may occur, constituting the so-called anthrax carbuncles. Such subacute conditions are especially found among horses, which are by nature not so susceptible to the disease as cattle and sheep.

On *post mortem* examination of an ox dead of anthrax, the most noticeable feature—one which has given the name “splenic fever” to the disease—is the enlargement of the spleen, which may be two or three times its natural size. It is of dark-red colour, and on section the pulp is very soft and friable, sometimes almost diffuent. A cover-glass preparation may be made from the spleen and stained with watery methylene-blue. On examination it will be found to contain enormous numbers of bacilli

mixed with red corpuscles and leucocytes, chiefly lymphocytes and the large mononucleated variety (Fig. 108). Pieces of the organ may be hardened in absolute alcohol, and sections cut in paraffin. These are best stained by Gram's method. Microscopic examination of such shows that the structure of the pulp is considerably disintegrated, whilst the bacilli swarm throughout the organ, lying irregularly amongst the cellular elements. The



FIG. 108.—Scraping from spleen of guinea-pig dead of anthrax, showing the bacilli mixed with leucocytes, etc. (Same appearance as in the ox.)  
“Corrosive film” stained with carbol-thionin-blue.  $\times 1000$ .

liver is enlarged and congested, and may be in a state of acute cloudy swelling. The bacilli are present in the capillaries throughout the organ, but are not so numerous as in the spleen. The kidney is in a similar condition, and here the bacilli are chiefly found in the capillaries of the glomeruli, which often appear as if injected with them. The lungs are congested and may show catarrh, whilst bacilli are present in large numbers throughout the capillaries, and may also be found in the air cells, probably as the result of rupture of the capillaries. The blood throughout the body is usually fluid and of dark colour.]

The lymphatic system generally is much affected. The glands, especially the mediastinal, mesenteric, and cervical glands, are enlarged and surrounded by oedematous tissue, the lymphatic vessels are swollen, and both glands and vessels may contain numberless bacilli. The heart may be in a state of cloudy swelling, and the blood in its cavities contains bacilli, though in smaller numbers than that in the capillaries. The intestines are enormously congested, the epithelium more or less desquamated, and the lumen filled with a bloody fluid. From all the organs the bacilli can be easily isolated by stroke cultures on agar.

It is important to note the existence of great differences in susceptibility to anthrax in different species of animals. Thus the ox, sheep (except those of Algeria, which only succumb to enormous doses of the bacilli), guinea-pig, and mouse are all very susceptible, the rabbit slightly less so. The last three are of course most used for experimental inoculation. We have no data to determine whether the disease occurs among these in the wild state. Less susceptible than this group are the horse, deer, goat, in which the disease occurs from time to time in nature. Anthrax also occurs epidemically in the pig, often from the ingestion of the organs of other animals dead of the disease. It is, however, doubtful if all cases of disease in the pig described on clinical grounds as anthrax are really such, and a careful bacteriological examination is always advisable. The human subject may be said to occupy a medium position between the highly susceptible and the relatively immune animals. The white rat is highly immune to the disease, while the brown rat is susceptible. Adult carnivora are also very immune, and the birds and amphibia are in the same position.

With these differences in susceptibility there are also great variations in the pathological effects produced in the natural or artificial disease. This is especially the case when we consider the distribution of the bacilli in the bodies of the less susceptible animals. Instead of the widespread occurrence described above, they may be confined to the point where they first gained access to the body and the lymphatic system in relation to it, or may be only very sparsely scattered in organs such as the spleen (which is often not enlarged), the lungs, or kidneys. Nevertheless the cellular structure of the organs even in such a case may show changes, a fact which is important when we consider the essential pathology of the disease.

*Experimental Inoculation.*—Of the animals commonly used in laboratory work, white mice and guinea-pigs are the most susceptible to anthrax, and are generally used for test inocula-

tions. If a small quantity of anthrax bacilli be injected into the subcutaneous tissue of a guinea-pig a fatal result follows, usually within two days. *Post mortem* around the site of inoculation the tissues, owing to intense inflammatory œdema, are swollen and gelatinous in appearance, small hæmorrhages are often present, and on microscopic examination numerous bacilli are seen. The internal organs show congestion and cloudy swelling, with

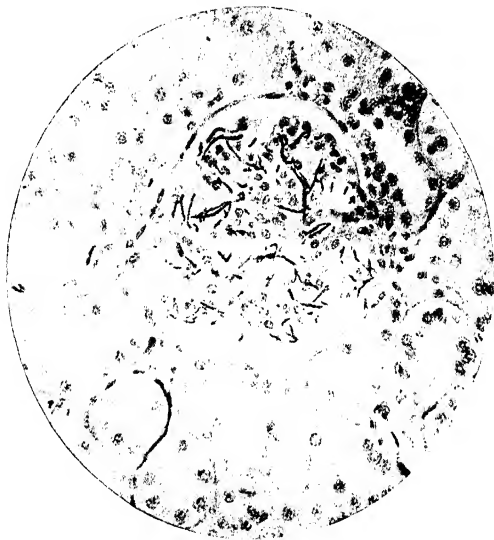


FIG. 109.—Portion of kidney of a guinea-pig dead of anthrax, showing the bacilli in the capillaries, especially of the glomerulus.

Paraffin section; stained by Gram's method and Bismarck-brown.  $\times 300$ .

sometimes small hæmorrhages, and their capillaries contain enormous numbers of bacilli, as has already been described in the case of the ox (Fig. 109); the spleen also shows a corresponding condition. Highly susceptible animals may be infected by being made to inhale the bacilli or their spores, and also by being fed with spores, a general infection rapidly occurring by both methods.

**Anthrax in the Human Subject.**—As we have noted, man occupies a middle position in the scale of susceptibility to anthrax. It is always communicated to him from animals, and



usually is seen among those whose trade leads them to handle the carcasses or skins of animals which have died of the disease. It occurs in two principal forms, the main difference between which is due to the site of entrance of the organism into the body. In one, the path of entrance is through cuts or abrasions in the skin, or through the hair follicles. A local condition called a "malignant pustule" develops, which may lead to a general infection. This variety occurs chiefly among butchers and those who work among hides (foreign ones especially). In Britain the workers of the latter class chiefly liable are the hide-porters and hide-workers in South-Eastern London. In the other variety of the disease the site of infection is the trachea and bronchi, and here a fatal result almost always follows. The cause is the inhalation of dust or threads from wool, hair, or bristles, which have been taken from animals dead of the disease, and which have been contaminated with blood or secretions containing the bacilli, these having afterwards formed spores. This variety is often referred to as "woolsorter's disease," from its occurring in the centres of the woolstapling trade (in England, chiefly in Yorkshire), but it also is found in places where there are hair and brush factories.

(1) *Malignant Pustule*.—This usually occurs on the exposed surfaces—the face, hands, fore-arms, and back, the last being a common site among hide-porters. One to three days after inoculation a small red painful pimple appears, soon becoming a vesicle, which may contain clear or blood-stained fluid, and is rapidly surrounded by an area of intense congestion. Central necrosis occurs and leads to the malignant pustule proper, which in its typical form appears as a black eschar often surrounded by an irregular ring of vesicles, these in turn being surrounded by a congested area. From this pustule as a centre subcutaneous œdema spreads, especially in the direction of the lymphatics; the neighbouring glands are enlarged. There is fever with general malaise. On microscopic section of the typical pustule, the central eschar is noticed to be composed of necrosed tissue and degenerating blood cells; the vesicles are formed by the raising of the stratum corneum from the rete Malpighi. Beneath them and in their neighbourhood the cells of the latter are swollen and œdematous, the papillæ being enlarged and flattened out and infiltrated with inflammatory exudation, which also extends beneath the centre of the pustule. In the tissue next the eschar necrosis is commencing. The subcutaneous tissue is also œdematous, and often infiltrated with leucocytes. The bacilli exist in the periphery of the eschar and in the neigh-

bouring lymphatics, and, to a certain extent, in the vesicles. It is very important to note that widespread œdema of a limb, enlargement of neighbouring glands, and fever may occur while the bacilli are still confined to the immediate neighbourhood of the pustule. Sometimes the pathological process goes no further, the eschar becomes a scab, the inflammation subsides, and recovery takes place. In the majority of cases, however, if the pustule be not excised, the œdema spreads, invasion of the blood stream may occur, and the patient dies with, in a modified degree, the pathological changes detailed with regard to the acute disease in cattle. In man the spleen is usually not much enlarged, and the organs generally contain few bacilli. The actual cause of death is therefore the absorption of toxins. It may here be said that early excision of an anthrax pustule, especially when it is situated in the extremities, is followed, in a large proportion of cases, by recovery.

(2) *Woolsorter's Disease*.—The pathology of this affection was worked out in this country especially by Greenfield. The local lesion is usually situated in the lower part of the trachea or in the large bronchi, and is in the form of swollen patches in the mucous membrane often with hæmorrhage into them. The tissues are œdematous, and the cellular elements are separated, but there is usually little or no necrosis. There is enormous enlargement of the mediastinal and bronchial glands, and hæmorrhagic infiltration of the cellular tissue in the region. There are pleural and pericardial effusions, and hæmorrhagic spots occur beneath the serous membranes. The lungs show collapse and œdema. There may be cutaneous œdema over the chest and neck, with enlargement of glands, and the patient rapidly dies with symptoms of pulmonary embarrassment, and with a varying degree of pyrexia. It is to be noted that in such cases, though numerous bacilli are present in the bronchial lesions, in the lymphatic glands and affected tissues in the thorax, comparatively few may be present in the various organs, such as the kidney, spleen, etc., and sometimes it may be impossible to find any.

(3) Infection occasionally takes place through the intestine, probably by ingestion of spores as in the case of animals; but this condition is rare. In such cases there is a local lesion in the intestinal mucous membrane, of similar nature to that in the bronchial form, the central parts of the hæmorrhagic areas being, however, sometimes necrotic and yellowish, and there is a corresponding affection of the mesenteric glands. In a case of this kind, recently recorded by Teacher hæmorrhagic meningitis,

associated with the presence of the bacilli in large numbers, occurred as a complication.

**The Toxins of the Bacillus Anthracis.**—Various theories were formerly held as to the mode in which the anthrax bacillus produces its effects. One of the earliest was the mechanical, according to which it was supposed that the serious results were produced by extensive blocking of the capillaries in the various organs by the bacilli. According to another, it was supposed that the bacilli used up the oxygen of the blood, thus leading to starvation of the tissues. The discovery of definite toxins which accounted for the pathogenic effects of certain bacteria led to such bodies being sought for in connection with the anthrax bacillus. Among other workers, Sidney Martin investigated this subject. This observer used alkali-albumin on which to grow the bacillus, this medium approaching most closely to the environment of the latter when growing in the animal body. From cultures in this medium, concentrated by evaporation either at 100° C. or in vacuo at 35° to 45° C., there were isolated proto-albumose, deuterio-albumose, and traces of peptone. The albumoses differed from those which occur in ordinary digestion, in being strongly alkaline in their reaction. This alkalinity, Martin held, was due to traces of an alkaloidal body of which the albumoses were the precursors, and which were formed when the process of digestion of the alkali-albumin by the bacillus was allowed to go on further. By the albumoses and the alkaloid, pathogenic effects were produced in animals, closely similar to those produced by the bacilli themselves. Martin, to account for the symptoms of the disease, considered that the fever was mostly due to the albumoses, while the œdema and congestion were due to the alkaloid which acted as a local irritant. He showed that prolonged boiling destroyed the activity of the albumoses, but not that of the alkaloid. Further, from the body fluids of animals dead of anthrax he isolated poisonous bodies similar to those produced by the bacilli growing in this artificial medium. Hankin and Westbrook arrived at the conclusion that the bacillus anthracis produces a ferment which, diffusing out into the culture fluid, elaborates albumoses from the proteids present in it. The bacilli also produce albumoses directly without the intervention of a ferment. Marmier, after cultivating the *b. anthracis* in peptone solution containing certain salts, removed all the albumoses from the resultant liquid, and from them, either by dialysis or extraction with glycerin, isolated a body which gave no reactions of albuminoid matter, peptone, propeptone, or alkaloid. This he considered the

toxin. It killed animals susceptible to anthrax by a sort of cachexia, and in suitably small doses could be used to immunise them against subsequent inoculation with virulent bacilli. It was chiefly retained within the bacilli when these were growing in the most favourable conditions. Unlike the toxins of tetanus and diphtheria, and unlike ferments, it was not destroyed by heating to 110° C. The toxin produced by the *b. anthracis* growing in a fluid medium remains intimately associated with the bacterial protoplasm, as such cultures when filtered are relatively non-toxic.

It cannot be said that great light has been thrown on the pathology of the disease by these researches. The effects of infection by the *b. anthracis* are those shared by all other organisms producing inflammation, the tendency to œdema production of an unwonted degree being the chief special feature and one with reference to which Martin's work may be important. That toxic effects do occur in anthrax is undoubted, for frequently, while the bacilli are still locally confined, there may occur pyrexia and œdema spreading widely beyond the pustule, but we have no definite information as to how these effects are produced. In this disease we are probably dealing with another example of the action of intracellular toxins, regarding which, as in other cases, little is known.

**The Spread of the Disease in Nature.**—We have seen that the *b. anthracis* rarely, if ever, forms spores in the body, and if the bacilli could be confined to the blood and tissues of carcasses of animals dying of the disease, it is certain that anthrax in an epidemic form would rarely occur. For it has been shown by many observers that in the course of the putrefaction of such a carcase the anthrax bacilli rapidly die out, and that after ten days or a fortnight very few remain. But it must be remembered that while still alive, an animal is shedding into the air by the bloody excretions from the mouth, nose, and bowel, myriads of bacilli which may rapidly spore, and thus arrive at a very resistant stage. These lie on the surface of the ground and are washed off by surface water. At certain seasons of the year the temperature is, however, sufficiently high to permit of their germination, and also of their multiplication, as they can undoubtedly grow on the organic matter which occurs in nature. They can again form spores. It is in the condition of spores that they are dangerous to susceptible animals. In the bacillary stage, if swallowed, they will be killed by the acid gastric contents; but as spores they can pass uninjured through the stomach, and gaining an entrance into the intestine, infect its

associated with the presence of the bacilli in large numbers, occurred as a complication.

**The Toxins of the Bacillus Anthracis.**—Various theories were formerly held as to the mode in which the anthrax bacillus produces its effects. One of the earliest was the mechanical, according to which it was supposed that the serious results were produced by extensive blocking of the capillaries in the various organs by the bacilli. According to another, it was supposed that the bacilli used up the oxygen of the blood, thus leading to starvation of the tissues. The discovery of definite toxins which accounted for the pathogenic effects of certain bacteria led to such bodies being sought for in connection with the anthrax bacillus. Among other workers, Sidney Martin investigated this subject. This observer used alkali-albumin on which to grow the bacillus, this medium approaching most closely to the environment of the latter when growing in the animal body. From cultures in this medium, concentrated by evaporation either at 100° C. or in vacuo at 35° to 45° C., there were isolated proto-albumose, deutero-albumose, and traces of peptone. The albumoses differed from those which occur in ordinary digestion, in being strongly alkaline in their reaction. This alkalinity, Martin held, was due to traces of an alkaloidal body of which the albumoses were the precursors, and which were formed when the process of digestion of the alkali-albumin by the bacillus was allowed to go on further. By the albumoses and the alkaloid, pathogenic effects were produced in animals, closely similar to those produced by the bacilli themselves. Martin, to account for the symptoms of the disease, considered that the fever was mostly due to the albumoses, while the cedema and congestion were due to the alkaloid which acted as a local irritant. He showed that prolonged boiling destroyed the activity of the albumoses, but not that of the alkaloid. Further, from the body fluids of animals dead of anthrax he isolated poisonous bodies similar to those produced by the bacilli growing in this artificial medium. Hankin and Westbrook arrived at the conclusion that the bacillus anthracis produces a ferment which, diffusing out into the culture fluid, elaborates albumoses from the proteids present in it. The bacilli also produce albumoses directly without the intervention of a ferment. Marnier, after cultivating the *b. anthracis* in peptone solution containing certain salts, removed all the albumoses from the resultant liquid, and from them, either by dialysis or extraction with glycerin, isolated a body which gave no reactions of albuminoid matter, peptone, propeptone, or alkaloid. This he considered the

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wall, and ultimately reach, and multiply in the blood. It is known that in the great majority of cases of the disease in sheep and oxen, infection takes place thus from the intestine. It was thought by Pasteur that worms were active agents in the natural spread of the disease by bringing to the surface anthrax spores. Koch made direct experiments on this point, and could get no evidence that such was the case. He thinks it much more probable that the recrudescence of epidemics in fields where anthrax carcasses have been buried is due to persistence of spores on the surface which has been infected by the cattle when alive.

**The Disposal of the Carcasses of Animals dead of Anthrax.**—It is extremely important that anthrax carcasses should be disposed of in such a way as to prevent their becoming future sources of infection. If anthrax be suspected as the cause of death no *post mortem* examination should be made, but only a small quantity of blood removed from an auricular vein for bacteriological investigation. If such a carcass be now buried in a deep pit surrounded by quicklime, little danger of infection will be run. The bacilli being confined within the body will not spore, and will die during the process of putrefaction. The danger of sporulation taking place is, of course, much greater when an animal has died of an unknown disease which on *post mortem* examination has proved to be anthrax, but similar measures for burial must be here adopted. In some countries anthrax carcasses are burned, and this, if practicable, is of course the best means of treating them. The chief source of danger to cattle subsequently, however, proceeds from the infection of fields, yards, and byres with the offal and the discharge from the mouths of anthrax animals. All material that can be recognised as such should be burned along with the straw in which the animals have lain. The stalls or buildings in which the anthrax cases have been must be limewashed. Needless to say, the greatest care must be taken in the case of men who handle the animal or its carcass that they have no wounds on their persons, and that they thoroughly disinfect themselves by washing their hands, etc., in 1 to 1000 solution of corrosive sublimate, and that all clothes soiled with blood, etc., from anthrax animals be thoroughly boiled or steamed for half an hour before being washed.

**The Immunising of Animals against Anthrax.**—Having ascertained that there was ground for believing that in cattle one attack of anthrax protected against a second, Pasteur (in the years 1880-82) elaborated a method by which a mild form of the disease could be given to animals, which rendered harmless a subsequent inoculation with virulent bacilli. He found that the continued growth of anthrax bacilli at 42° to 43° C. caused them to lose their capacity of producing spores, and also gradually to lose their virulence, so that after twenty-four days they could no longer kill either guinea-pigs, rabbits, or sheep. Such cultures constituted his *premier vaccin*, and

protected against the subsequent inoculation with bacilli which had been grown for twelve days at the same temperature, and the attenuation of which had therefore not been carried so far. The latter constituted the *deuxième vaccin*. It was further found that sheep thus twice vaccinated now resisted inoculation with a culture which usually would be fatal. The method was to inoculate a sheep on the inner side of the thigh by the subcutaneous injection, from a hypodermic syringe, of about five drops of the *premier vaccin*; twelve days later to again inoculate with the *deuxième vaccin*; fourteen days later an ordinary virulent culture was injected without any ill result. This method was applicable also to cattle and horses, about double the dose of each vaccine being here necessary. Extended experiments in France generally confirmed earlier results, and the method was, before long, used to mitigate the disease, which in many departments was endemic and a very great scourge. Since that time the method has been regularly in use. It is difficult to arrive at a certain conclusion as to its merits. Undoubtedly a certain number of animals die of anthrax either after the first or second vaccination, or during the year following vaccination. At the end of a year the immunity is lost in about 40 per cent of the animals vaccinated; and thus to be permanently efficacious the process would have to be repeated every year. Further, the immunity is much higher in degree if, after the first and second vaccinations, an inoculation with virulent anthrax is performed. Everything being taken into account, however, there is no doubt that the mortality from natural anthrax is much diminished by this system.

During the twelve years 1882-93 3,296,815 sheep were vaccinated, with a mortality, either after the first or second vaccination, or during the subsequent twelve months, of '94 per cent, as contrasted with the ordinary mortality in all the flocks of the districts of 10 per cent. During the same time 438,824 cattle were vaccinated, with a mortality of '34 per cent, as contrasted with a probable mortality of 5 per cent if they had been unprotected.

The immunisation of animals against anthrax has always been found to be a difficult proceeding. The most usual technique has been to commence with Pasteur's vaccines, and to follow these by careful dosage with virulent cultures. Marchoux in this way produced immunity, and found that the serum of immune animals had a certain degree of protective and curative action. The most successful attempts in this direction have been those of Sclavo and of Sobernheim. The former observer, after trying various animals, came to the conclusion that the



ass was the most suitable. He first employed a method similar to that of Marchoux; later, however, after noting the effects of the serum of an animal so immunised, he commenced the immunisation by injecting 5 to 15 c.c. of this serum along with a slightly attenuated culture of the bacilli. A few days later this was followed up with injections of virulent cultures which could now be periodically introduced for many months, and a high degree of immunity resulted. What was even more important, the serum of such an animal had strongly protective and curative properties. It has been extensively used in the treatment of anthrax in man. In a case of malignant pustule 30 to 40 c.c. are injected in quantities of 10 c.c. into the abdominal wall, and if necessary the injection is repeated on the following day. In cases treated by Selavo himself the serum is alone employed, and its action is not aided by the excision of the pustule usually practised. The results obtained have been very good,—Selavo, out of 164 cases, had only ten deaths or about a fourth of the ordinary mortality in Italy. Sobernheim independently elaborated an almost identical method of combining passive with active immunisation for the obtaining of a powerful anti-serum, and he has used this for the protective inoculation of cattle. The technique is to inject the serum into one side of the neck or into one thigh and the culture (Pasteur's second vaccine) into the other side; the doses given are for cattle or horses 5 c.c. of serum and .5 c.c. culture, and for sheep 4 c.c. of serum and .25 c.c. culture. The method has been widely used in Germany and in Brazil, and its originator claims as its advantages simplification of application, in that one operation instead of two is sufficient, less risk of death following the immunisation procedure, and higher degree and more lasting character of the immunity resulting. Whether this method is really more efficient than that of Pasteur future experience will show, but it might be preferable for developing protection in herds at a time when an epidemic was raging. During the development of active immunity it is likely in every case (see Immunity) that there is a period of increased susceptibility to the disease. Such a period would be more likely to occur with the Pasteur method than with the Sobernheim procedure, where the presence in the animal's body of the protective serum might tide it over the stage when the action of the vaccine was lowering its resistance.

The effects of the *b. anthracis* have been much studied with a view to the shedding of light on the processes obtaining in resistance and the development of immunity. Many puzzling

facts have long been known; for example, in the dog, which shows great natural resistance, the serum has little if any bactericidal action, while in the susceptible rabbit there is present a serum capable of killing the organism. Such observations have hitherto been without explanation. Again the properties of the serum of immune animals have been much discussed. Sobernheim and others have been unable to detect in it any trace of special bactericidal action. Selavo found that the serum when heated to  $55^{\circ}$  C. did not lose its protective properties; as the serum might have been complemented (see Immunity) by the serum of the animal into which it was injected, he simultaneously introduced an anti-complementary serum and found that the heated serum was still effectual. From this he deduces that in the action of the serum substances of the nature of immune body and complement are not concerned. Many have thought that the serum had a stimulating effect on the leucocytes, but Cler has brought forward ground for supposing that its effect is a sensitising one on the bacteria, and that thus the effects are to be traced to opsonic action. With regard to the formation of the protective substances, it is stated that the spleen and bone-marrow are richer in these than the blood fluids. In this connection an interesting fact may be mentioned, namely, that Roger and Garnier found evidence of the liver and spleen having special capacities for killing anthrax bacilli; an otherwise fatal dose could be introduced into the portal vein or the splenic artery without causing death.

**Methods of Examination.**—These include (a) microscopic examination; (b) the making of cultures; and (c) test inoculations.

(a) *Microscopic Examination.*—In a case of suspected malignant pustule, film preparations should be made from the fluid in the vesicles or from a scraping of the incised or excised pustule, and stained with a watery solution of methylene-blue and also by Gram's method. By this method practically conclusive evidence may be obtained; but sometimes the result is doubtful, as the bacilli may be very few in number. In all cases confirmatory evidence should be obtained by culture. Occasionally bacilli are so scanty that both film preparations made from different parts and even cultures may give negative results, and yet a few bacilli may be found when a section of the pustule is examined. It should be noted that the greatest care ought to be taken in manipulating a pustule before excision, as the diffusion of the bacilli into the surrounding tissues may be aided and the condition greatly aggravated. The examination

of the blood in cases of anthrax in man usually gives negative results, with the exception of very severe cases, when a few bacilli may be found in the blood shortly before death, though even then they may be absent.

(b) *Cultivation*.—A small quantity of the material used for microscopic examination should be taken on a platinum needle, and successive strokes made on agar tubes, which are then incubated at 37° C. At the end of twenty-four hours anthrax colonies will appear, and can be readily recognised from their wavy margins by means of a hand lens. They should also be examined microscopically by means of film preparations.

(c) *Test Inoculations*.—A little of the suspected material should be mixed with some sterile bouillon or water, and injected subcutaneously into a guinea-pig or mouse, or it may be introduced into the subcutaneous tissue by means of a seton. If anthrax bacilli are present, the animal usually dies within two days, with the changes in internal organs already described.

## CHAPTER XIV.

### TYPHOID FEVER—BACILLI ALLIED TO THE TYPHOID BACILLUS.

*OTHER NAMES.*—ENTERIC FEVER: GASTRIC FEVER. *GERMAN*,  
TYPHUS ABDOMINALIS: ABDOMINALTYPHUS: UNTERLEIBS-  
TYPHUS. *FRENCH*, LA FIÈVRE TYPHOÏDE.

**Introductory.**—The organism now known as the bacillus typhosus was first described in 1880-1 by Eberth, who observed its microscopic appearances in the intestinal ulcers and in the spleen in cases of typhoid fever. It was first isolated (from the spleen) in 1884 by Gaffky, and its cultural characters were then investigated. In 1885 Escherich observed a bacillus, now known as the bacillus coli communis, which occurs in the normal intestine and which both microscopically and culturally closely resembles the typhoid bacillus. Ordinarily the b. coli is no doubt a harmless saprophyte, but under experimental conditions in animals and also naturally in man it may manifest pathogenic properties. Investigation has shown that these two bacilli belong to a widespread group of organisms isolated from various disease conditions, which all bear close resemblances to one another and whose differentiation is often a matter of considerable difficulty. Other members of this group are the paratyphoid bacillus, the organism of bacillary dysentery, the b. enteritidis of Gaertner, the psittacosis bacillus, and the bacillus of hog cholera.

**Bacillus Typhosus.**—*Microscopic Appearances.*—It is sometimes difficult to find the typhoid bacilli in the organs of a typhoid patient. Numerous sections of different parts of a spleen, for example, may be examined before a characteristic group is found. The best tissues for examination are a Peyer's patch where ulceration has not yet commenced or where it is

just commencing, the spleen, the liver, or a mesenteric gland. The spleen and liver are better than the other tissues named, as in the latter the presence of the *b. coli* is more frequent. From scrapings of such solid organs dried films may be prepared and stained for a few minutes in the cold by any of the strong staining solutions, *e.g.* with carbol-thionin-blue, or with Ziehl-

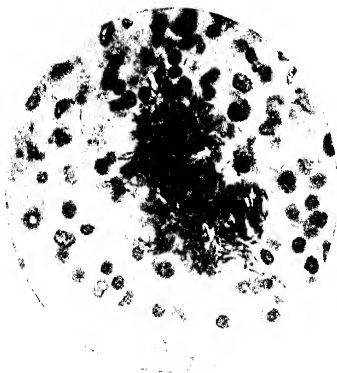


FIG. 110.—A large clump of typhoid bacilli in a spleen. The individual bacilli are only seen at the periphery of the mass. (In this spleen enormous numbers of typhoid bacilli were shown by cultures to be present in a practically pure condition.) Paraffin section; stained with carbol-thionin-blue.  $\times 500$ .

Neelsen carbol-fuchsin diluted with five parts of distilled water. As a rule decolorising is not necessary. For the proper observation of the arrangement of the bacilli in the tissues, paraffin sections should be prepared and stained in carbol-thionin-blue for a few minutes, or in Löffler's methylene-blue for one or two hours. The bacilli take up the stain somewhat slowly, and as they are also easily decolorised, the aniline-oil method of dehydration may be used with advantage (*vide* p. 93). In such preparations the characteristic appearance to be looked for is the occurrence of

groups of bacilli lying between the cells of the tissue (Fig. 110). The individual bacilli are  $2\ \mu$  to  $4\ \mu$  long, with somewhat oval ends, and  $.5\ \mu$  in thickness. Sometimes filaments  $8\ \mu$  to  $10\ \mu$  long may be observed, though they are less common than in cultures. It is evident that one of the short oval forms may frequently in a section be viewed endwise, in which case the appearance will be circular. This appearance accounts for some, at least, of the coccus-like forms which have been described. The bacilli are decolorised by Gram's method.

**Isolation and Appearances of Cultures.**—To grow the organism artificially it is best to isolate it from the spleen, as it exists there in greater numbers than in the other solid organs, and may be the sole organism present even some time after death. The spleen is removed whole, and a portion of its

capsule is seared with a cauterly to destroy all superficial contaminating organisms. A small incision is made into the organ with a sterile knife, a little of the pulp removed by a platinum needle, and agar or gelatin plates are prepared, or successive strokes made on agar tubes. On the agar media the growths are visible after twenty-four hours' incubation at 37° C. On agar plates the superficial colonies are thin and film-like, circular or slightly irregular at the margins, dull white by reflected light, bluish-grey by transmitted light. Colonies in the substance of the agar are small, and appear as minute round points. When viewed under a low objective, the surface colonies are found to be very transparent (requiring a small diaphragm for their definition), finely granular in appearance, and with a very coarsely crenated and well-defined margin. The deep colonies are usually spherical, sometimes lenticular in shape, and are smooth or finely granular on the surface, and more opaque than the superficial colonies. On making cover-glass preparations, the bacilli are found to present the same micro-

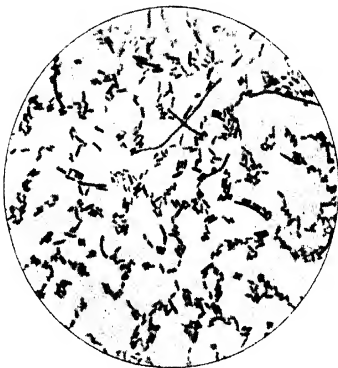


FIG. 111.—Typhoid bacilli, from a young culture on agar, showing some filamentous forms.

Stained with weak carbol-fuchsin.  $\times 1000$ .

scopic appearances as are observed in preparations from solid organs, except that there may be a greater number of the longer forms which may almost be called filaments (Fig. 111). The same is true of films made from young gelatin colonies. Sometimes the diversity in the length of the bacilli is such as to throw doubt on the purity of the culture. Its purity, of course, can be readily tested by preparing plates from it in the usual way. As a general rule in a young (twenty-four to forty-eight hours old) colony, grown at a uniform temperature, the bacilli are plump, and the protoplasm stains uniformly. In old cultures, or in cultures which have been exposed to changes of temperature, the protoplasm stains only in parts; there may be an appearance of irregular vacuolation either at the centre or at the ends of the bacilli. There

is no evidence that spore-formation occurs in the typhoid bacillus.

*Motility*.—In hanging-drop preparations the bacilli are found to be actively motile. The smaller forms have a darting or rolling motion, passing quickly across the field, whilst some show rapid rotatory motion. The filamentous forms have an undulating or serpentine motion, and move more slowly. Hanging-drop preparations ought to be made from agar or broth cultures



FIG. 112.—Typhoid bacilli, from a young culture on agar, showing flagella. Stained by Van Ermengem's method.  $\times 1000$ .

not more than twenty-four hours old. In older cultures the movements are less active.

*Flagella*.—On being stained by the appropriate methods (*vide* p. 103) the bacilli are seen to possess many long wavy flagella which are attached all along the sides and to the ends (Fig. 112). They are more numerous, longer, and more wavy than those of the *b. coli*.

*Characters of Cultures*.—Stab cultures in *peptone gelatin* give a somewhat characteristic appearance. On the surface of the medium growth spreads outwards from the puncture as a thin

film or pellicle, with irregularly wavy margin (Fig. 113, A). It is semi-transparent and of bluish-white colour. Ultimately this surface growth may reach the wall of the tube. Not infrequently, however, the surface growth is not well marked. Along the stab there is an opaque whitish line of growth, of finely nodose appearance. There is no liquefaction of the medium, and no formation of gas. In stroke cultures there is a thin bluish-white

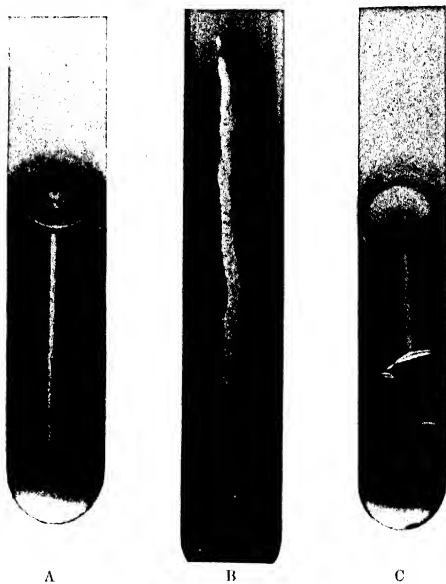


FIG. 113.

- A. Stab culture of the typhoid bacillus in gelatin, five days' growth.  
B. Stroke culture of the typhoid bacillus on gelatin, six days' growth.  
C. Stab culture of the bacillus coli in gelatin, nine days' growth; the gelatin is split in its lower part owing to the formation of gas.

film, but it does not spread to such an extent as in the case of the surface growth of a stab culture (Fig. 113, B). In gelatin plates also the superficial and deep colonies present corresponding differences. The former are delicate semi-transparent films, with wavy margin, and are much larger than the colonies in the substance, which appear as small round points (Fig. 114). These appearances, which are well seen on the third or fourth day, resemble those seen in agar plates, as already described in the method of isolation; but on gelatin the surface colonies are